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CANADIAN THESES ON MICROFICHE / THÈSES CANADIENNES SUR MICROFICHE

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TITLE OF THESIS/*TITRE DE LA THÈSE* PREPARATION AND EVALUATION OF ELECTROLYTICALLY RADIOIODINATED BLEOMYCINS

UNIVERSITY/*UNIVERSITÉ* UNIV. OF ALBERTA

DEGREE FOR WHICH THESIS WAS PRESENTED/*GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE* M Sc RADIOPHARMACY

YEAR THIS DEGREE CONFERRED/*ANNÉE D'OBTENTION DE CE GRADE* 1975

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PREPARATION AND EVALUATION OF ELECTROLYTICALLY RADIOIODINATED BLEOMYCINS

by

ROBERT G. BROWN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF

MASTER OF SCIENCE

IN

RADIOPHARMACY

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

EDMONTON, ALBERTA

FALL, 1975

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled PREPARATION AND EVALUATION OF ELECTROLYTICALLY RADIOIODINATED BLEOMYCINS submitted by Robert G. Brown in partial fulfilment of the requirements for the degree of Master of Science in Radiopharmacy.

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Date August 21/1975

TO DEANA, TRACEY, AND CAROL

ABSTRACT

A method was developed for the electrolytic radioiodination of the anti-tumor drug, bleomycin. The bleomycin A_2 sub-fraction, one of the most tumor specific and least toxic of the complex components, was isolated and purified from the commercially available bleomycin complex (BLENOXANE^R), using a silica gel column chromatograph followed by a gel exclusion technique. The various parameters involving the electrolytic method for radioiodination of bleomycin A_2, as well as the total complex, were studied. Predictable yields of up to 91% of the radioiodinated product were routinely obtained. Stability studies of the ^{125}I-iodobleomycins indicated thermal stability at 100°C with no appreciable dehalogenation occurring for 30 days at 20°C. Thin layer chromatographic analysis did not suggest the presence of any molecular degradation.

Tissue distribution studies in a mouse model bearing a solid form of a Ehrlich ascites tumor, exhibited maximum tumor:blood and tumor:muscle ratios of 9.5:1 and 19:1 respectively six hours after administration. There was no significant difference in tumor uptake of the ^{125}I labeled bleomycin complex or ^{125}I labeled A_2 sub-fraction. The tumor:blood and tumor:muscle ratios of radioactivity obtained with radioiodinated bleomycins were noticeably superior to those of a ^{99m}Tc-bleomycin complex at the same time interval.

(R - Bristol Laboratories Dorval, Quebec)

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. A. Shysh, Dr. L. Wiebe, and Dr. A. Noujaim for their encouragement and guidance throughout this project.

I would also like to say a special thank you to Mr. Chris Ediss for his help throughout this work, and Mr. Andrew Stevens for his interest and willingness to help.

The assistance of the Dr. W.W. Cross Cancer Institute, and in particular, Dr. B. Lentle, is gratefully acknowledged.

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ABSTRACT

A method was developed for the electrolytic radioiodination of the anti-tumor drug, bleomycin. The bleomycin A_2 sub-fraction, one of the most tumor specific and least toxic of the complex components, was isolated and purified from the commercially available bleomycin complex (BLENOXANE^R), using a silica gel column chromatograph followed by a gel exclusion technique. The various parameters involving the electrolytic method for radioiodination of bleomycin A_2, as well as the total complex, were studied. Predictable yields of up to 91% of the radioiodinated product were routinely obtained. Stability studies of the ^{125}I-iodobleomycins indicated thermal stability at 100°C with no appreciable dehalogenation occurring for 30 days at 20°C. Thin layer chromatographic analysis did not suggest the presence of any molecular degradation.

Tissue distribution studies in a mouse model bearing a solid form of a Ehrlich ascites tumor, exhibited maximum tumor:blood and tumor:muscle ratios of 9.5:1 and 19:1 respectively six hours after administration. There was no significant difference in tumor uptake of the ^{125}I labeled bleomycin complex or ^{125}I labeled A_2 sub-fraction. The tumor:blood and tumor:muscle ratios of radioactivity obtained with radioiodinated bleomycins were noticeably superior to those of a ^{99m}Tc-bleomycin complex at the same time interval.

INTRODUCTION

Although the ideal tumor imaging agent has yet to be discovered, recent developments in the field of nuclear medicine have produced agents and techniques that are more sensitive and more specific to delineate neoplasm. The majority of research has been directed toward the discovery of radiopharmaceuticals that are selectively taken up by many types of malignant tissue but not to any appreciable degree by normal nonneoplastic tissue. The most widely used material of this type in tumor diagnostics is ^{67}Ga gallium citrate. This agent has the ability to concentrate in soft tissue tumors[1] but has also been shown to accumulate in a wide variety of benign sites.[2]

With the discovery of the bleomycin complex by Umezawa et al.[3] in 1966 this polypeptidic antibiotic has come under close scientific scrutiny. A high degree of selective uptake and anti-neoplastic activity initially qualified this complex as a promising chemotherapeutic agent.[3]

Of the nineteen sub-fractions that compose the complex, extensive studies indicated that the A_2 sub-fraction, which accounts for 55 to 70% of the total complex, was more tumor specific and did not show the kidney damage that is prevalent with the accompanying B fractions.[4]

The complex exists as a natural copper chelate and this property of the molecule raised the possibility of chelating not only copper, but other cations as well. This characteristic made the bleomycin available to nuclear medical procedures if a suitable radionuclide could be incorporated into the structure. Initially ^{57}Co was chelated

to the bleomycin complex, due to its availability.[5] This product proved to be stable *in vivo* and exhibited good imaging qualities but the physical properties of the nuclide were undesirable and alternatives were sought.

Thakur and his associates[6] successfully chelated ^{111}In to bleomycin complex and this radiopharmaceutical has received a great deal of attention as a clinical diagnostic agent for tumor imaging. One of the drawbacks with ^{111}In-bleomycin is the apparent *in vivo* instability. During biological metabolism the chelate bond appears to be broken, with the subsequent binding of the free nuclide to transferrin and ultimate deposition occurring in the bone marrow.

Very recent studies, using a ^{99m}Tc complexing, have resulted in much the same results.[7] High blood pool background made imaging difficult, particularly in the abdominal and thoracic region, and there is evidence that the ^{99m}Tc dissociates from the bleomycin and is associated with the albumin blood fractions.[6]

It seemed logical therefore, in view of these findings, that if a covalently bonded radionuclide could be introduced into the most desirable of the bleomycin sub-fractions (i.e. A_2), a tumor specific and biologically stable agent could be developed. In the near future, with the probable ready availability of ^{125}I, which has excellent physical characteristics for scintigraphic detection, the feasibility of radioiodination of bleomycin A_2 sub-fraction was undertaken.

A method using silica gel column chromatography was developed and resulted in the separation and purification of the A_2 sub-fraction from the commercial complex. Extensive chromatographic techniques

were evolved to maintain the quality control of the unlabeled bleomycins and which later served as assay methods for radiochemical purity of the radiolabeled bleomycin. Although many documented methods for polypeptide iodinations are available, the electrolytic technique of Rosa and Pennisi[8] was chosen.

Radioiodinated bleomycin A_2 sub-fraction of high percentage yield was subsequently obtained and a preliminary animal tissue distribution study was undertaken. The results were encouraging indicating a preferential uptake of the ^{125}I-bleomycin in malignant tissue of tumor model mice. A more comprehensive distribution study was then undertaken. A portion of the results of this project were reported at the Annual Meeting of the Canadian Association of Nuclear Medicine in Winnipeg, Manitoba, January 1975.

SURVEY OF THE LITERATURE

I. Bleomycin Complex

A. History of Bleomycin Complex

In 1956 Maeda and Umezawa[9] isolated, from a strain of streptomyces verticillus, a blue copper-containing gluco-polypeptide which they named phleomycin. This complex exhibited a high anti-bacterial activity and subsequent biological studies on mouse and rat tumor models showed a selective anti-neoplastic action.[10] This anti-tumor mechanism was determined by Talaschi and Kornberg[11] to be a selective inhibition of DNA polymerase and a resultant cessation of DNA synthesis. It was also found, however, after further in-depth investigation, that the phleomycin complex also caused a high degree of irreversible nephrotoxicity. An alternate antibiotic was then sought that would have the anti-tumor activity of phleomycin but be less toxic.

While investigating new strains of actinomycetes, isolated from soil samples obtained from a coal mine in Kaho-Gun, Japan, a new complex of antibiotics produced by a strain of strept. verticillus was isolated. It could be differentiated from phleomycin by its stability in acid or alkali and also by its Rf values on paper chromatography. The complex was named bleomycin.[3]

The bleomycin complex was a basic water soluble gluco-polypeptide, pale blue in color, and occurred as a natural copper chelate in the hydrochloride salt form. The copper could be removed by treatment with 8-hydroxyquinolone. Bleomycin was found to contain a mixture of fractions with a molecular weight of approximately 1400 and was

methanol soluble but practically insoluble in acetone, ethanol and ether.[3]

B. Isolation and Purification of Bleomycin

After culturing, the broth containing the complex was adjusted to pH 4.6, filtered and the filtrate applied to an Amberlite IRC 50 (H^+ form) column (5.5 cm diameter, 1750 ml vol). After washing the column with distilled water, it was eluted with .2N HCl and the bleomycin fractions collected, pooled and neutralized with Amberlite IR-413 (OH^- form). The neutralized eluate was concentrated, washed with acetone and dried in vacuo. The resultant powder was then extracted with methanol and the methanol-insoluble portion discarded. The methanol solution was concentrated, dried to powder and washed with ethanol. The ethanol insoluble portion was dried and contained the crude bleomycin complex.[3]

Alumina column chromatography was utilized as the next step in purification of the crude extract. This process was eventually deleted, however, as the material obtained from this procedure had a decreased therapeutic index against ascites Ehrlich carcinoma in mice. It was evident that structural changes were occurring during alumina column chromatography.[12]

A Sephadex G-25 (200 ml, 2.0 cm diameter) column was eluted with distilled water and the fractions collected in 5 ml aliquots and lyophilized. This procedure also effectively divided the complex into two general fractions labeled A and B. The A fraction eluted

off the column first. Subsequent paper chromatography, using Toyo filter paper No. 51 and developing in 10% ammonium chloride by the ascending method, to 30 cm, indicated two fractions as well. The A fraction had R_f values of .88-.99, the B .66-.70 on paper.[12]

The Sephadex G-25 eluate containing the complex was concentrated, dried to powder, and re-dissolved in warm methanol. Any insoluble residue was discarded and to the resultant solution, ethanol was added which resulted in a fine precipitate being formed. This precipitate was then filtered out of the solution, isolated and dried to powder. It contained pure bleomycin complex. Prior to subjecting the complex to a more detailed analysis to determine the various components of the A and B fraction numerous experiments were run to calculate the composition of the material.

The complex was found to contain sulfur, indicating cystine or cysteine, or methionine moieties.[3] Bleomycin gave positive Pauly reactions, indicative of imidazole or histidine entities, positive Ehrlich reaction (tryptophane) and positive Dragendorf (quaternary ammonia). All the A fraction gave a negative Sakaguchi reaction while the B fraction gave a positive reaction. This indicates the presence of a guanadino group or arginine.

The elemental analytical data (A - C 45.2%, H 6.84%, N 15.43%, Cl 9.32%, S 1.45%), (B - C 42.06%, H 6.52%, N 15.09%, Cl 12.24%, S .267%) confirmed the peptide nature of the components.[3] The ultra-violet absorption spectra indicated a shoulder at 243-244 nm (mμ) and a maximum at 289-290 nm. This indicates the presence of aromatic structures.

The basic differences between phleomycin and bleomycin were resolved as well. On paper chromatography developed in 10% ammonium chloride all the phleomycin values fall below R_f .81 and were thus different from the A fraction (R_f .88-.99). The B fraction was more difficult to differentiate from phleomycin by paper chromatography but may be separated by the difference in the optical density of ultraviolet absorption at 289-295 nm. The bleomycin complex, both A and B fractions, proved to be much more stable than phleomycin in alkaline (pH 9) or acid (pH 2) solutions[4] at 22°C.[12]

The final step in the purification and isolation of the various sub-fractions contained in the A and B fractions was carried out using gradient column chromatography.[12] The eluate from the Sephadex G-25 column, after being dried to powder, was dissolved in 0.1M ammonium formate and applied to a 2 x 35 cm CM-Sephadex C-25 column. Linear gradient elution was carried out by increasing the concentration of the ammonium formate from 0.1M to 1.0M. The effluent optical density was continuously read at 253.7 nm and the fractions collected. All fractions containing the same substance were combined and lyophilized.

The copper-chelated A fraction was shown to contain six sub-fractions designated A_1, A_2, A_3, A_4, A_5, A_6. The A_2 component was the main sub-fraction. Similarly the B fraction contained five sub-fractions designated B_1, B_2, B_3, B_4, B_5 in the order they were eluted. The B_2 and B_4 were the main components of the B fraction.[12] Subsequent closer investigation using CM-Sephadex C-25 gradient column chromatography has distinguished five new components which are present in the complex.[13] This was accomplished by re-chromatographing the eluates

from each major sub-fraction. The new sub-fractions were designated demethyl A_2, A_2'-a, A_2'-b, A_2'-c, B_1' and B_6.

When spotted on Silicagel G (Merck) and developed with 10% ammonium acetate:methanol (1:1), the various sub-fractions all have characteristic R_f values and thus identification was simplified. It is also interesting to note that all the B complex gave a negative Ninhydrin reaction (free amino group) as did the A_1, A_2 and A_3 fractions.

The new components acted, in some cases, quite differently than expected. Table 1 shows a comparison of the R_f of the components on paper developed with 10% ammonium chloride and tlc developed with 10% ammonium acetate:methanol (1:1) as well as the various peptide identification reactions.[12,13]

The A_2 fraction is the main component of the complex, accounting for 55-70% of the total volume. B_2 accounts for approximately 20%, A_1 5% and A_2' 5%. The rest of the sub-fractions are present in very small, almost negligible amounts. The commercial preparation available in Canada (Blenoxane-Bristol Laboratories) contains purified copper free bleomycin complex.

C. Structural Analysis of Bleomycin

Further investigation was carried out by Umezawa and his associates to determine the structure and peptide sequence of bleomycin. Most of the original work was conducted on the major fraction A_2 (copper chelated).[14]

Table 1

Characteristic Behavior of Bleomycin Complex Chromatographically[12,13]

Component	R_f paper Chromatography (ppc)	R_f Thin layer Chromatography (tlc)	Ninhydrin Reaction	Sakaguchi Reaction
A_1	.92	.74	-	-
A_2	.83	.40	-	-
Demethyl A_2	.71	.80	-	-
A_2'-a	.82	.70	+	-
A_2'-b	.82	.70	+	-
A_2'-c	.71	.71	-	-
A_3	.85	.13	-	-
A_4	.85	.49	+	-
A_5	.86	.51	+	-
A_6	.88	.30	+	-
B_1	.71	.75	-	+
B_1'	.73	.80	-	-
B_2	.72	.68	-	+
B_3	.71	.68	-	+
B_4	.72	.60	-	+
B_5	.70	.52	-	+
B_6	.52	.47	-	+

Bleomycin A_2 was hydrolized with 6N HCl at 105°C for twenty hours in a sealed tube. The hydrolysate contained at least seven ninhydrin positive products. Each component was isolated by ion exchange (Dowex 50 WX4) and designated as compounds I, II, III, IV, V, VI, VII in order of elution.

The molecule, on partial hydrolysis, yielded two peptides initially which were designated Tetrapeptide A and Tripeptide S. Tetrapeptide A, on total acid hydrolysis, yielded four amino acids, but only two peptide bonds and therefore is correctly a pseudo-peptide. Tripeptide S contained three amino acid components.[14] Figure 1 illustrates the total structure of bleomycin A_2 determined by selective cleavage by N-bromosuccinimide.[15]

1) Tetrapeptide A

Compound V was identified as L-β-aminoalanine by comparison of infrared spectrum and optical rotation with an authentic sample. Compound II was shown to contain a β-lactam ring with the formula β-amino β-(4-amino-6-carboxyl-5-methylpyrimidin-2yl) propionic acid. Compound IV was originally identified as β-carboxy-histidine but further investigation revealed the structure to be β-hydroxyhistidine.[16,17] This was accomplished and confirmed by detailed nuclear magnetic resonance (nmr) comparison to pure β-hydroxy histidine. As this component is of special interest, a more detailed scrutiny will appear later. Compound III was identified as 4-amino-3-hydroxy-2-methyl-n-valeric acid.

2) Tripeptide S

The first eluate from the ion exchange separation of the acid hydrolysis of the complex was the first amino acid in the tripeptide S. It was shown to be L-threonine by its chromatographic behavior, infrared spectrum and optical rotation.[14]

Compound VI proved to be the peptide which contributed to the maximum u.v. absorption at 290-295 nm. This compound appeared to be the portion of the bleomycin B molecule which differentiated it from the previously discovered phleomycin.[18,19] The bleomycin structure contained a bithiazole chromophore component whereas the phleomycin (D_1 fraction) contained only one thiazole ring. It was identified as 2'-(2 aminoethyl)-2,4'bithiazole-4-carboxylic acid. The terminal end of the tripeptide containing compound VII was determined to contain different distinguishable amine groups in the various sub-fractions of bleomycin.

It was also discovered that by adding amines to the fermentation broth of the streptomyces verticillus, these amines were incorporated in the terminal amine position of the bleomycin.[20,21] This method was utilized to not only increase the proportions or yield of a particular sub-fraction in the complex but also to produce new semi or biosynthetic bleomycins. On the addition of particular diamines or triamines, it suppressed almost completely the production of other natural bleomycins. In this manner Umezawa and his associates were able to produce forty-two artificial bleomycins and study their biological activity. For

example, by supplying the terminal amine of the A_2 sub-fraction to the culture media a product containing 82% A_2 was produced. This made available larger quantities of some of the minutely present sub-fractions for further testing and purification.[20]

In figure 1a, representative terminal amine groups are shown.[20] The guanidino group is evident in the B-complex thus the positive Sakaguchi reactions.

On the selective cleavage of bleomycin A_2 by N-bromosuccinimide a peptide linkage was shown joining compound III and the L-theonine of compound I.[15]

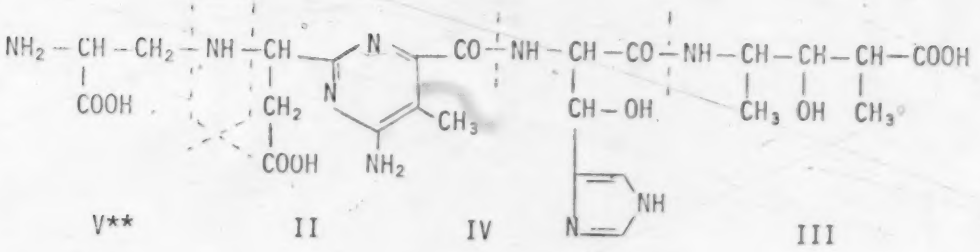
Compound IV of bleomycin A_2 contains β-hydroxy histidine. The stereochemistry of this entity has been determined and was shown to be L-erythro-β-hydroxyhistidine by X-ray crystallography.[17] One of the outstanding features of this structure was the conformation around the C-2 and C-3 bond. The imidazole was gauche to the carboxyl group and trans to the amino group, while the hydroxyl group was gauche to both. Apparently the introduction of the hydroxyl group on the β-carbon (C-3) caused the imidazole to be in an unusual position in comparison to that found in various other histidine-containing peptides.[17]

The imidazole ring is positively charged and the five ring atoms and C-3 are nearly coplanar. This whole configuration caused the imidazole ring to project from the molecule rather than being buried within the structure. The β-hydroxyl group has now been established as the active binding site for the sugar linkage and probably due to the heterocyclic charge on

Figure 1

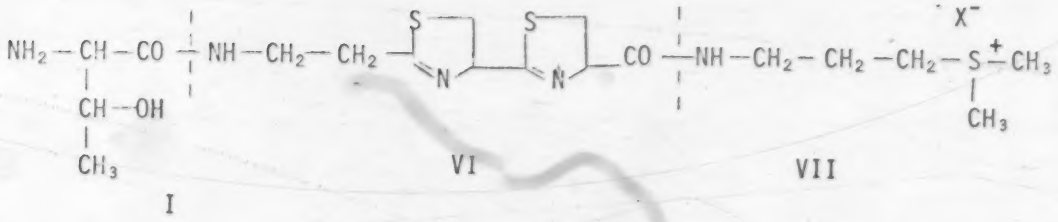
Structure of Bleomycin A_2 Sub-fraction[15]

Tetrapeptide A



V** II IV III

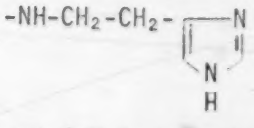
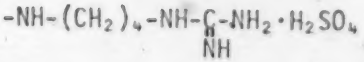
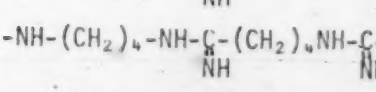
Tripeptide S



I VI VII

Figure 1a

Terminal Amine Components of Bleomycin Complex Sub-fractions[20]

A_1	$-NH-CH_2-CH_2-CH_2SO-CH_3-\frac{1}{2}H_2SO_4$
Demethyl A_2	$-NH-CH_2-CH_2-CH_2-S-CH_3 \cdot HCl$
A_2	$-NH-CH_2-CH_2-CH_2-\overset{+}{S}(CH_3)_2 \cdot Cl^- \cdot HCl$
A_2'-a	$-NH-(CH_2)_4NH_2 \cdot 2HCl$
A_2'-b	$-NH-(CH_2)_3NH_2 \cdot 2HCl$
A_2'-c	
B_2	
B_4	
A_5	$-NH-(CH_2)_4-NH-(CH_2)_4NH_2 \cdot 3HCl$
A_6	$-NH-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_4NH_2 \cdot 4HCl$

the imidazole ring, plays a major role in the chelating ability of this molecule.[17]

D. Antibacterial Activity of Bleomycin

The antibacterial effects of each of the copper chelated natural sub-fractions of bleomycin against various bacterial stains were studied.[4] Each of the A and B fraction's activity against Staph aureus (209 P), K. pneumonia, S. lutea, B. subtilis (PCI 219), E. coli, S. typhosa (No. 63), S. flexneri (EW2), Mycobacterium 607 and M. phlei were tested by the agar streak method. In the A fraction, of those components which give a positive ninhydrin reaction, (A_4, A_5 and A_6) A_5 and A_6 showed the strongest antibacterial activity. Among the A bleomycins which exhibited a negative ninhydrin reaction (A_1, A_2, A_3) the A_3 sub-fraction was the most active. It is interesting to note that the sub-fractions that eluted off the CM-Sephadex C-25 column at the highest molar concentration had the greatest antibacterial activity. All the B fractions gave negative ninhydrin, and showed a weaker activity than any of the A fraction. All the results indicated that the bleomycin complex has a broad-spectrum of antibacterial activity.[4]

E. Distribution and Toxicity of Bleomycin in Animals

Many biological studies in both animals and humans have been carried out using both the copper chelated and the copper-free bleomycin. The copper-free preparations have identical biological and distribution patterns as the natural copper chelated form.

Certain advantages were found with the copper-free materials, however, making them the more desirable compounds.[23] There was a marked decrease in the toxicity of bleomycin in mice when injected in the copper-free form. There also appeared to be an increase in the vascular permeability with the copper chelated forms of bleomycin A_2 or B_2. This caused venous damage at the site of injection and may be related to some of the nephro-toxic effects noted with copper-bleomycin B_2. It was also found that the most prevalent of the sub-fractions, the A_2, was more completely excreted in the urine, after eight hours, as the copper-free entity.[22]

The toxicity of the individual bleomycin sub-fractions was directly related to their distribution in various organs or tissue. There was also a delayed or accumulative toxicity observed, as was noticed with many anti-tumor substances. To eliminate or decrease this effect of additive toxicity, biweekly injections were suggested,[4] because even though bleomycin is rapidly excreted, (85% in twenty-four hours) it can still be detected in the urine after seventy-two hours. This factor was born out by studies in mice and rabbits. The LD50 was determined to be 150-200 mg/kg using a single injection.[4] The survival rate was prolonged considerably if the total dose was fractionated over a period of time. The gross systemic effects of an injection of bleomycin A complex in rabbits was loss of appetite and subsequent weight decrease, and an increase in leukocytes in the blood.[22]

In sub-acute toxicity studies on mice conducted by Matsuda[23] et al, using bleomycin A complex (copper chelated), the results paralleled those of previous investigators and it was concluded that the grade and frequency of

toxic manifestations were closely related to dose level and injection period. These experiments also indicated the transient weight loss and other primary toxic symptoms were more prolonged in male animals and in senile animals. In the high dose animals (50 and 200 mg/kg) the animals exhibited mottled hyperemia of the lung, and slight liver, kidney and spleen damage on autopsy.[23]

Serial injections in dogs up to a total of 140 mg/kg of bleomycin A complex showed a transient weight decrease and decreased liver function which returned to normal after twenty-one days. Alopecia was noted as well as inflammation of the foot pads. The effects were dose-related and reversible on cessation of injections. The toxicity of bleomycin A was the same by either intravenous, intraperitoneal or subcutaneous injections.[23]

Original tissue distribution investigations carried out by Umezawa's group were carried out with the copper chelated A fractions.[4] Injections of 50 mg/kg were administered to mice and showed high concentrations in kidney, lung and liver. Subsequent studies by the same group two years later were much more detailed in their findings. In this experiment the various sub-fractions were injected in the copper chelated form and compared to the copper-free A_2 and B_2 compounds.[22] The following data show the detailed distribution and accumulation in mice injected with a dose of 50 mg/kg. In the copper containing group, the A_2 showed the highest skin concentration, which accounted for the alopecia and dermal inflammation noted previously. The peritoneum concentration was high for A_1, A_2, A_5, B_2 and B_4. Surprisingly low concentrations of all the sub-fractions were noted in the liver and spleen three hours

post-injection. Kidney concentrations were high with all the sub-fractions except for the A_2 component. The B_4 compound had the highest kidney accumulation, and follow-up studies on this material exhibited severe kidney damage in dogs.[4] The concentration in lung tissue was relatively high with A_2, B_1 and B_4 and in particular B_1 and B_4.[22] In the uterus, again, the B_4 sub-fraction showed the highest levels. From these results the A_2 and A_5 showed the highest urine levels after three hours and thus were considered to be the most rapidly excreted. As reported earlier, the copper-free A_2 was excreted even more rapidly.

It could be seen that due to the elevated accumulation in the skin and lung that these locations were the sites of the greatest activity and toxicity. Umezawa also reported reversible alopecia, nail discoloration and deformation and inflammation and tenderness of the skin in dogs.[24] The transient liver impairment and weight loss was also noted. In these preliminary animal studies it was also noted that there was an absence of haematopoetic depression.[23] This proved to be a very important factor in the later clinical usage in humans. Most patients with malignant disease have depressed marrow function due to previous chemotherapeutic agents or radiation therapy, and the need for a non-myelotoxic agent was important.

F. Toxicity of Bleomycin in Humans

The first reported clinical trials using bleomycin in humans were conducted in Japan in 1969. Ichikawa et al[25] noted a high incidence of skin toxicity and a slight occurence of fever and anorexia. Most

importantly, they related a small but definite incidence of pulmonary fibrosis at total dose levels in excess of 300 mg. More recent reports on toxicity, using copper-free bleomycin complex supplied by Bristol Laboratories, Syracuse, N.Y., have been conducted in the United States. A typical batch of this material contains 53% A_2, 12.2% A_1, 6.6% A_2', 1.7% A_5, 4.4% B_1, 19.9% B_2 and 2.2% B_4. Yagoda[26] reported that all patients had an occurrence to some extent of dermal or mucosal manifestations. These vary from a mild erythemia or striae to deep ulcerations, particularly in areas of maximum pressure. Mild fever, chills, vomiting and fatigue were also noted. The dosage schedule in this study of 274 patients on a daily basis of .25 mg/kg of body weight rather than biweekly injections. The results seemed to be related to not only the dose of bleomycin used, but also on patient condition. Alopecia occurred in all patients to varying degrees as did transient liver impairment. The most important finding, however, was the evidence of pulmonary fibrosis in patients who had received accumulated doses of approximately 250 mg. In some cases this condition could be reversed by large doses of prednisone (100 mg/day).[26] Rudders[27] also reported serious pulmonary complications in 10.4% of the cases of bleomycin treated patients. It was fatal in 2.6% of the patients and on autopsy they showed dense interstitial fibrosis and pneumonitis. Most of the pulmonary toxicity again proved to be dose related with a sharp increase in incidence at doses in excess of 300 mg total.

G. Antineoplastic Activity of Bleomycin

1) Animals

A multitude of researchers have investigated the anti-tumor activity of bleomycin, either as a complex or as individual sub-fractions. Umezawa, in his original work, noted the inhibition of both sarcoma 180 and Ehrlich ascites tumor in mice.[3] The cytotoxicity of each bleomycin was determined by Ishizuka using compounds in their natural copper chelated form.[4] This study was accomplished by introducing two million (2×10^6) Ehrlich ascites cells intraperitoneally to mice and then injecting sub-lethal doses of each sub-fraction of bleomycin daily. The survival time was then recorded compared to control tumor-bearing animals. The A_5 had the strongest activity against this particular tumor, followed by A_3 and A_2. The A fraction bleomycins proved to be much more effective and less toxic.[4]

The A complex as a whole was also scrutinized against Ehrlich carcinoma, both ascites type and solid tumor form, as well as sarcoma 180. At a daily dose of 3.12 mg/kg over a period of ten days a 77% inhibition of all the types of tumor was noted.[4] At 25 mg/kg a 94.2% inhibition was noted, however this was considered double the maximum tolerable dose in mice and the toxic effects of the drugs were strongly prevalent. Yoshida rat sarcoma cells in tissue culture were inhibited 91% in the presence of 65 mg/ml. It is of special interest that the toxicity studies carried out by Ishizuka on dogs bearing spontaneous tumors, culminated in the

disappearance of a vaginal lymphosarcoma after ten 6 mg/kg injections administered biweekly.[4]

Further distribution studies carried out furnished additional evidence of the high skin and lung concentrations and particularly high tumor uptake in neoplasms located in the epithelial tissue.[4]

It was established that the least toxic and the most tumor specific of the sub-fractions was the copper-free A_2 and A_5.[22] These compounds showed much greater inhibitory action against Ehrlich carcinoma in mice and were excreted more rapidly than either the copper-free B complex or the counterpart copper-chelated preparations. These observations suggested that the copper-free bleomycins could be considered for treatment of squamous cell or epithelial malignancies in humans.

2) Humans

Ichikawa[25] and his co-workers, in the initial clinical trials, noted the unusual effectiveness of bleomycin in carcinoma of the penis and scrotum. They also noted response of 33% to 78% in squamous cell carcinomas of the skin, lung, head and neck.

Further successful results were reported by Yagoda[26] in the treatment of not only carcinoma of the head, neck, and lymphosarcomas, but a significant response was noted in over 50% of a group of patients with advanced Hodgkins disease. He also pointed out the absence of any myelosuppression.

Rudder's[27] study on malignant lymphomas also shows a 49% response and concluded bleomycin had a significant anti-tumor activity against these neoplasms, particularly Hodgkins disease.

A complete clinical review was undertaken in 1972 by the U.S. National Cancer Institute[28] and the data on 1,174 bleomycin treated patients was completely evaluated. This data was collected from world wide sources, the largest group coming from Ichikawa in Japan. In this paper, Blum concludes that as a chemotherapeutic agent the bleomycin complex would provide a significant benefit to patients with lymphoma, testicular tumors and squamous cell carcinoma of certain anatomical sites refractory to conventional therapy and when further conventional therpy is prevented by myelotoxicity.[28]

H. Mechanism of Action of Bleomycin

Suzuki et al[29,30,31] have determined the mechanism of action of bleomycin using the total complex, copper-free A_2 sub-fraction and copper chelated A_2 on growing cells of E. coli, Ehrlich carcinoma, and HeLa cells. It was observed that all three of the compounds acted in a similar manner and caused inhibition of DNA and protein synthesis, the DNA synthesis being more profoundly affected than the protein synthesis.[29] RNA or tRNA synthesis was not interfered with to any extent. The activity of bleomycin was highly dependent on the concentration of cells and also phosphate in the medium, being more pronounced at low phosphate concentrations and with fewer cells.[29]

It was concluded that bleomycin causes single strand scission of DNA to a much greater degree than double strand breaks. This was determined by sucrose gradient centrifugation analysis. It was found that the presence of a sulfhydryl compound (2-mercaptoethanol) was

necessary for this reaction[30] and the activity was inhibited by the addition of millimolar amounts of metallic divalent cations (Cu^{++}, Co^{++}, Zn^{++}) or EDTA.[31] This indicates that the chelating activity of bleomycin is necessary for its activity and actually competes with other chelating agents and they weakent the biological action.

The interaction of bleomycin with DNA is unique in two points when compared to other drugs in this field:[31]

a) It causes a decrease in the melting temperature (Tm) of DNA, others cause an increase.

b) Bleomycin is the only drug to cause single strand scission, the others have a greater affinity for double strand helical structures.

Of course, the over-all effect of this single strand scission is the inhibition of incorporation of thymidine into DNA and mitosis.

II. Tumor Scanning with Radionuclide Labeled Bleomycin

The ultimate goal of all these extensive studies were to investigate the efficacy of this tumor specific agent, with its minimal toxicity, as a chemotherapeutic agent. Due to the physical characteristics of its structure and its rather uncomplicated molecule, another complete discipline was to utilize this drug. The natural chelating ability of bleomycin has formed the basis for complexing a number of radioactive nuclides to the molecule for use as a non-invasive tumor diagnostic agent by gamma scintigraphy.

Making use of the natural chelating ability of bleomycin led Renault and his group in France to a complete investigation of the chelating capacity of the molecule.[32] It appears that bleomycin preferentially chelates the divalent metals and in particular, those belonging to the transition metal group. Of the following group of metals studied, Cu^{+2}, Co^{+2}, Zn^{+2}, Ni^{+2}, Hg^{+2}, Fe^{+2}, Sr^{+2}, Ag^{+2}, Fe^{+3}, Yb^{+3}, Tc, Rd, In^{+3}, Ga^{+3}, and Au^{+4}, it was not surprising to find that copper and zinc, and to a lesser extent nickel and cobalt, were chelated preferentially and to the highest degree of stability. The data presented shows that bleomycin has a chelating capacity of 26 µg of Cu^{+2}/mg, 25 µg Zn^{+2}/mg, 17 µg Co^{+2}/mg, 15 µg of Ni^{+2}/mg, 9.5 µg Hg^{+2}/mg and all other cations less than 1.0 µg/mg. Although copper and zinc isotopes would then be the most desirable, the difficulties in availability and in their physical decay characteristics, would certainly cause problems in routine usage.

A. Cobalt 57 Bleomycin

The most available nuclide that would fall into this group of transition metals was ^{57}Co. Novel and Renault[5] were the first group to publish reports on the use of radiolabeled bleomycin as a possible tumor scanning agent. They carried out a complete clinical study on ten patients, with previously diagnosed malignancies, using ^{57}Co bleomycin. The investigation was very encouraging, permitting the precise assessment of the diagnosed neoplasms plus the discovery of primary sites in two cases and four cases of undiagnosed metastases. The ^{57}Co bleomycin showed a very rapid plasma clearance with only

2-3% remaining in the blood pool twenty-four hours post-injection. The urinary excretion was equally as rapid with greater than 50% of the activity collected after twenty-four hours. This urine activity was determined to be ^{57}Co bleomycin, not free ^{57}Co, by cation exchange resin chromatography. It was found, however, that 1-2% of the activity remained fixed in the liver and kidney, suggesting the presence of free ^{57}Co. The researchers concluded that the chelate was being broken down and the ^{57}Co was released from the bleomycin during a biological degradation process.[5]

It was also noted in this study that the more active or malignant the neoplastic tissue, the greater the uptake and fixation of the drug. This fact is born out by the many studies that have since been published. It is speculated it may be due to the vasculatory of the particular tumor. Tumor to tissue ratios were three to one with ^{57}Co bleomycin and this allowed detection and visualization of tumors of 1 cm in size. Practically all types of carcinomas were imaged to some degree, the best visualization being in the lymphoma or lymph node metastases group. Little success was noted with sarcomas.[5]

The great disadvantage of ^{57}Co, of course, was the physical characteristics of the isotope. With a half-life of 270 days, waste disposal becomes a problem and if, in fact, the cobalt was being released from the bleomycin molecule during metabolism, there would be a considerable patient hazard.

A more recent clinical study was carried out by Moret[33] and his co-workers, on eighteen patients, to diagnose and localize various epitheliomas, malignant melanomas and their metastases. Surprisingly,

since bleomycin shows a high uptake in the skin, the ^{57}Co-bleomycin complex failed to give consistent fixation at the level of tumor cells investigated. They suggested that the value of this labeled compound remains to be proven and a further extensive examination was needed.

An animal study conducted in Japan using a semiconductor detector compared the uptake of ^{57}Co-bleomycin and ^{67}Ga-citrate in Ehrlich tumor-bearing mice and also in experimentally induced inflammatory condition.[34] A resume of the findings showed the tumor uptake was more rapid with the ^{57}Co-bleomycin and reached a maximum at six hours and was then excreted very rapidly. The ^{67}Ga-citrate, on the other hand, reached a maximum in twenty-four hours and was very slowly excreted. Although the accumulation in tumor was higher with the ^{67}Ga-citrate, the tumor to non-tumor levels of ^{57}Co-bleomycin were always superior and therefore thought to be more specific. The authors postulated that the accumulation mechanism of the two compounds governs the different uptake profiles. The ^{67}Ga was found to localize in the mitochondria and microsomal fractions in the cytoplasm while the ^{57}Co-activity was mainly associated with the nuclei attached to the DNA molecules.[34]

B. Indium 111 Bleomycin

Thakur and Merrick reported, at the same symposium as Renault, the use of an ^{111}I-bleomycin complex and some of its pharmacological aspects.[6]

The indium 111 was a much more suitable isotope than ^{57}Co with a half-life of sixty-seven hours and nearly ideal gamma emissions of

179 and 247 KEV with no beta component. The isotope was produced at the cyclotron facility at Hammersmith Hospital in London, by the 30 MeV alpha particle bombardment of natural silver. After ion exchange separation the ^{111}In was obtained carrier free in the chloride salt form.[35]

The radionuclides of bivalent elements have been shown to be chelated by bleomycin preferentially and a simple mixing together will produce a complex under normal conditions (22°C, neutral pH).[32] Trivalent elements, however, are not chelated under these conditions. Ideal labeling was obtained when both the $^{111}InCl_3$ and the bleomycin were dissolved in known volumes of .9% sodium chloride and added together to give a theoretical yield of 1 mCi/mg. This solution is slightly acidic and after heating for thirty minutes a vitual 100% label is obtained.[35]

The thermal stability of the ^{111}In complex was determined by repeated chromatographic analysis at various time intervals, over a period of seven days at 22°C and 37°C.[32] No free indium activity was found. The same results were found after autoclaving at 121°C for thirty minutes.[6]

The complex bond was also challenged *in vitro* with some of the bivalent or trivalent ions that may be present *in vivo*, namely Cu^{+2}, Ca^{+2}, Fe^{+2} and Fe^{+3}. The chelate remained stable, with no free indium activity detected, indicating no exchange with other metals take place under experimental conditions.[6]

Blood clearance studies of ^{111}In-bleomycin in animals (Wistar rats) were compared to $^{111}InCl_3$. More than 95% of the administered

^{111}In-bleomycin activity was cleared from the circulating blood two hours post-injection whereas a very high percentage of $^{111}InCl_3$ activity was still present in the blood at this time.[6]

It is known that bleomycin is enzymatically inactivated biologically to varying degrees in different tissues or organs.[21] This could possibly be the reason that tumor tissue retains the nuclide. The mechanism suggested was that the isotope is deposited in the tissue after degradation, or that malignant tissue lacks the enzyme or enzymes to inactivate bleomycin. Attempts to isolate the enzyme have proven difficult due to its instability. This inactivation was especially noted in liver, kidney, marrow, and spleen with lower values in the skin and lung. There is evidence that a different enzyme may deactivate selected sub-fractions of bleomycin.[21]

It was not surprising, therefore, to find the nuclide being released from the ^{111}In-bleomycin at some point of the <u>in vivo</u> metabolism. Chromatographic evidence proved that ten minutes post-injection the indium activity was associated with the bleomycin but after four hours the indium activity was bound to the same protein fraction in the blood as is observed following the injection of free nuclide. This fraction was shown to be transferrin.[6] The subsequent fate was the deposition of the ^{111}In-transferrin in the bone marrow. Regardless of this short-coming, many extensive studies have been carried out on this complex. It is now available for routine clinical diagnosis in the United States under the trade name of Tumor Scintigraphin from Medi-Physics, Emeryville, California, at a specific activity of 2.5 mCi/mg.

1) Tissue Distribution in Animals

Initial distribution studies of ^{111}In-bleomycin in Walker tumor model rats, indicated substantial tumor uptake six hours post-injection.[6] The incorporation into slower growing tumors increased up to seventy-two hours post-injection while in tumors with a more rapid doubling time there was no significant increase in uptake after twenty-four hours. Varying the amount of bleomycin concentration per injector did not alter the uptake to any extent. There was, however, an advantage to using the highest specific activity possible. The activity in the tumor appeared to be fixed up to about six hours after injection while the blood levels diminished progressively. High specific activity would allow better images of localization as the tumor to blood ratios reflect in this study. High kidney and marrow activity was also noted, as expected.

Some interesting results from tumor mouse distribution using ^{111}In-bleomycin were reported in 1973. Using a solid form of the Ehrlich ascites tumor, Grove and Eckelman and Reba[36] were able to show very high uptake at one hour post-injection followed by a decrease in all tissues at four hours. Of significance was the increase of activity in the tumor at twenty-four hours. This increase was also noted in the liver, lung and skin. These results may be indicative of a different biological degradation from that of ^{57}Co-bleomycin. The authors did, however, conclude that ^{57}Co-bleomycin was a superior imaging agent. It cleared the blood and liver more rapidly than ^{111}In-bleomycin, and reached

much higher and more consistent tumor to blood and tumor to liver ratios after twenty-four hours. Due to the divalent metal it appeared to form a more stable chelate even when subjected to enzymatic degradation in the tissue. The question was now, to determine if the imaging potential of ^{111}I-bleomycin was indeed due to the bleomycin localizing in the tumor, or if in fact, the indium was being released from the bleomycin and localizing on its own as ionic indium, or possibly linked to transferrin.

Robbins and his coworkers carried out an extensive study on the ^{111}I-bleomycin and $^{111}InCl_3$ kinetics in mice, bearing a number of various transplantable tumors.[37] In this study they also duplicated Thakur's original work on the thermal and chemical stability of the complex. Contrary to Thakur, they found the ^{111}In-bleomycin complex to be completely unstable in the presence of excess Cu^{+2} ions and a complete exchange appeared to be taking place with the subsequent release of the indium.

Robbins et al. used an AKR strain of mice bearing Ridgeway osteogenic sarcoma and C5731/6 mice bearing Lewis lung tumor and B-16 melanoma. One group of Lewis lung tumor models were injected with ^{111}In-bleomycin, and 10 μCi of $^{111}InCl_3$. The mice were serially sacrificed at one, six, twenty-four and forty-eight hours post-injection and the various organs and tissues of interest were assayed. There was a remarkable similarity in the localization and distribution of the two agents after forty-eight hours. The tumor concentration with $^{111}InCl_3$ was considerably better than the ^{111}In-bleomycin, but the $^{111}ICl_3$ was cleared more

slowly from the blood. The highest tumor concentration was found at one hour but the best tumor to blood, and thus optimum scanning time, appeared to be from twenty-four to forty-eight hours for all the tumors investigated. The similarity between the two agents in their distribution seems to confirm that the ^{111}In-bleomycin dissociates *in vivo* and even suggests that $^{111}InCl_3$ may be a superior scanning agent.[37]

2) Clinical Studies with ^{111}In-Bleomycin

Due to the wide variance in animal studies and, even with the knowledge that ^{111}In-bleomycin seems to dissociate *in vivo*, the relative potential of it as a useful scanning agent seemed apparent. Clinical studies were carried out by a number of workers.

Thakur and Merrick[6] in 1972 studied twenty patients, during the initial ^{111}In-bleomycin investigations, with suspected brain tumors. They were able to distinguish between infarcts and tumors, and two secondary sites of malignancy were detected that had been missed using the conventional pertechnetate.

Verma *et al.*[36] studied the uptake of the commercially available radiopharmaceutical in 101 patients with various known primary or metastatic tumors. These included: malignant, melanomas, breast carcinomas, adenocarcinomas, sarcomas, lymphomas and epidermoid carcinomas. The ^{111}In-bleomycin scans were correlated to findings from routine clinical evaluations. Eighty of the 101 patients had detectable localization in malignant tissue. Eight sites of abnormal uptake were deemed as false positive as there was no evidence of neoplasm.

Grove and his group followed up their original animal work on ^{111}In-bleomycin with a clinical study on patients and directly compared tumor uptake of this complex with ^{57}Co-bleomycin and a tumor imaging agent that was gaining recognition, ^{67}Ga-citrate.[37] Attempts were also made in this experiment to chelate ^{67}Ga and ^{59}Fe to the bleomycin molecule but were unsuccessful.[36] Studies were completed on five patients, however, using ^{57}Co and ^{111}In-bleomycin and ^{67}Ga-citrate. They found the ^{67}Ga-citrate to provide better visualization of tumor but the ^{111}In-bleomycin gave variable results due to a cation contaminant.

A subsequent clinical evaluation of ^{111}In-bleomycin was also conducted using ^{57}Co and Ga-citrate for comparison by this group.[39] They concluded that due to some of the inherent disadvantages of ^{67}Ga-citrate, such as high gastrointestinal uptake obscuring the abdominal area, a twenty-four to seventy-two hour delay before imaging because of slow blood clearance and a low degree of sensitivity and specificity in certain tumors, labeled bleomycin, in particular, ^{57}Co-bleomycin, had distinct advantages as a tumor imaging agent. In their study of fifteen patients ^{57}Co-bleomycin detected 73% of the tumors evaluated while the ^{67}Ga-citrate agent visualized only 47%. Uptake was noted in a pyogenic inflammation by both agents, leading to false positive results. Results with the ^{111}In-bleomycin were quite poor and except for the physical disadvantages of ^{57}Co, it was a much superior radiopharmaceutical for tumor imaging. The whole body radiation dose was considerably smaller with the ^{57}Co and ^{111}In complex than with the ^{67}Ga-citrate (.03 and .15 Rads compared to .9 Rad).[39]

Goodwin and Lin[40] confirmed the stability of the ^{111}In-bleomycin chelate chromatographically, the positive uptake in squamous cell carcinoma, and also the appearance of bone marrow uptake on delayed forty-eight hour scans.

Most of the in-depth studies on commercially available ^{111}In-bleomycin have been carried out by Emmerson, O'Mara and Lillien[41] in Arizona including recent evaluation of the complex in pediatric oncology.[40] They reported the results on forty prior cases. More positive lesions were detected than with ^{67}Ga and the instance of false positives were much lower. They also noted that indium-bleomycin is not tumor specific but also localizes in clinical and experimental abscesses and fractures.[41]

This group also noted an overall correlation of 85% between abnormal ^{111}In-bleomycin accumulation and known sites of neoplastic involvement. The most precise imaging was for lymph and soft tissue disease sites. A 6% false negative value and 9% false positive value was noted. Multiple melanoma and leukemia failed to be detected and again progressively increasing marrow uptake was seen.[41]

An excellent review on the Current Status of Tumor Imaging by D. Lillien has appeared in the literature which sums up the efficacy of ^{111}In-bleomycin as a neoplastic scanning agent even with the known drawbacks.[42] He points out that although the routinely used ^{67}Ga-citrate localizes in some malignant tissue it is entirely nonspecific and has even been used to evaluate abscesses and osteomyelitic lesions. It gives a high percentage

of false negative and false positive scans consequently. A further drawback is in the nuclide itself; ^{67}Ga has a complex decay scheme with a number of gamma photons of varying energies but none in high abundance. In a direct comparison of this agent with ^{111}In-bleomycin in over 400 cases, the bleomycin complex was able to detect 90% of the disease sites; the ^{67}Ga-citrate 79%. Although the ^{111}In-bleomycin does not offer any advantage over ^{67}Ga with regard to specificity, it appears to detect a broader spectrum of tumors.

The pediatric onocological study carried out by this same group has shown the ^{111}I-bleomycin complex to be an acceptable diagnostic tool for tumor detection in children.[43] It was utilized in the detection and localization of various soft tissue primary and metastatic neoplasms, particularly of the neuroblastoma, lymphoma, rhabdomyosarcoma and Wilm's tumor types. The main advantages indicated were the high positive findings (83%), low false negatives (7%), low false positives (10%), and the low toxicity and safety of the compound. There was also noted a lack of significant bowel activity, which is important, as so many neoplasms in children involve the abdominal area.[43]

Horn et al.[44] have recently shown a 64% detection rate in visualizing malignant melanomas in fifty-one patients using ^{111}I-bleomycin.

C. Technetium-99m

In the original work carried out by Thakur on ^{111}I-bleomycin, comparative studies were conducted using a stannous chloride reduced ^{99m}Tc-bleomycin complex.[6] The ^{99m}Tc activity, however, was found to be associated almost completely with the albumin fraction of blood four hours post-injection, indicating a dissociation of the molecule in vivo. The liver uptake was also notably higher than the ^{111}In-bleomycin, and very low tumor to blood ratios also may have been due to leakage of the ^{99m}Tc into the blood pool after being released from the bleomycin molecule. The indium-bleomycin appeared to hydrolyze and remain in the tissues.[6] Results such as these did not discourage a host of researchers to investigate the possibility of forming a stable ^{99m}Tc-bleomycin complex. The advantages are obvious, with the ideal availability, physical characteristics, safety and cost of the ^{99}Tc. Various methods of preparing ^{99m}Tc-bleomycin have been attempted all with varying success.

Mori[45] reported on the usage of a ^{99m}Tc labeled bleomycin using stannous chloride-ascorbate complex with high specific activity. Very rapid excretion was noted, however imaging was successful at thirty minutes, with approximately 1% of the label accumulating in the tumor (fibrosarcoma), and .58% was recovered after twenty-four hours, in tumor bearing mice.

A total of 142 clinical cases were investigated and positive scintillation camera images were detected in 82% of the cases after only one hour post administration of intravenous doses.[45] These compared with a 65% detection rate using ^{67}Ga-citrate. It was also

possible to detect smaller malignant masses, and metastatic processes were much more favourably imaged with the ^{99m}Tc-bleomycin. The one main disadvantage was the high and rather diffuse activity in kidney, bladder, nasal area and the blood pool twenty-four hours following injection of ^{99m}Tc-bleomycin.

An electrolytic process has also been devised for the labeling of various ^{99m}Tc compounds, one of which is ^{99m}Tc-bleomycin, by Kato and his co-workers."[6] The end product is 95% labeled after an electrolysis time of twenty seconds at 10 mA. The labeled material appeared to have as good or better radiochemical and stability properties as those produced by conventional chemical methods. Unfortunately there were no follow-up biological studies on this preparation to date.

Lin and Goodwin[7] have carried out an extensive study on the labeling of bleomycin with ^{99m}Tc, using a simple tin reduction method, and the subsequent uptake and distribution in experimental animals and humans. They were able to obtain approximately a 50% label which was then purified to remove any unbound technetium by elution with .008N HCl through a AG1-X8 column. Tissue distribution in mice bearing a transplanted KHSS carcinoma in the flank revealed tumor to blood concentration levels of three to one after six hours while high activity in liver, kidneys, stomach, spleen and lungs were noted. The blood clearance was extremely rapid with an initial disappearance of 50% in twelve minutes. Eighteen cancer patients with biopsy-proven malignant lesions were scanned from one half hour to six hours after administration of the ^{99m}Tc-Sn-bleomycin.[7] A very high background was noted making scintigraphic detection very difficult. This was particularly true in the abdominal area.

A ^{99m}Tc labeling technique utilizing a stannous-pyrophosphate complex has been introduced by Bardy et al[47] in France and appears to be very successful. This method is used not only for the ^{99m}Tc labeling of bleomycin but also for "tagging" red blood cells. The reducing agent in this case is the stannous-pyrophosphate complex rather than the conventional stannous chloride. Although the nature of the technetium-bleomycin bond is unknown, this method allowed quantitative labeling depending on the amounts of bleomycin and tin used. The amounts of stannous chloride used in the stannous chloride-pyrophosphate reduction are considerably less than the amounts used in the standard reduction procedure using just stannous chloride, and the complex appears to be more strongly bonded and more stable. A 94.1% label was obtained and no purification step was necessary.[47]

D. Bleomycin Labeled with Radiocopper

The ability of bleomycin to preferentially chelate copper ions naturally, has led to the investigation of using various isotopes of this metal to form tumor imaging compounds. Coates and his group[48] used ^{67}Cu produced by the ^{67}Zn(n,p) ^{67}Cu reaction. They found a virtual 100% chelation possible. Plasma clearance, studied in dogs, exhibited almost identical values to that of ^{57}Co-bleomycin. There was no indication of free copper activity in vivo by way of increased liver uptake. Tumor distribution was not included in the report.

Ryo and Ice[49] in Michigan have also experimented with copper-bleomycin complex. Using ^{64}Cu a comparative study was undertaken using this nuclide chelated to bleomycin and ^{99m}Tc-bleomycin, along

with tritiated bleomycin, for complete distribution studies in Fisher rats bearing an endocrine "autonomous" breast tumor. The labeling procedures were assayed using chromatographic analysis and a near 100% label with the ^{64}Cu was obtained. At twenty-four hours the tumor uptake based on percent dose gram^{-1} of ^{64}Cu-bleomycin was twenty-four times that of ^{99m}Tc-bleomycin. It was again pointed out by the data of this experiment that bleomycin labeled with different nuclides behave differently biologically. The authors suggest that perhaps ^{67}Cu may be a better isotope to consider because of its longer half-life.[49]

E. Platinum 195m Labeled Bleomycin

Another interesting nuclide, that of platinum 195m, has recently been introduced into the bleomycin molecule.[50] Initial animals studies indicated an unusually high blood background, but the molecule remained intact in vivo and was excreted unmetabolized.

The results of all this research can be summed up quite concisely. Bleomycin appears to have as good as or perhaps better specificity than the current radionuclidic imaging agents used. The main disadvantages are two: At best the bleomycin labels have been chelates which are not particularly stable in vivo or in some cases in vitro, and the ideal nuclide has not been found to produce a stable yet easily detectable agent with the present scintigraphic methods. The ability to covalently bond a radioactive element, with good physical characteristics, to bleomycin would add a very valuable tool to the early diagnosis of malignant disease.

F. Radioiodinated Bleomycin

Denardo's group has recently successfully iodinated bleomycin using a modified iodine monochloride method.[51,52] They report that the iodination is very pH and time dependent but an 85% label was obtained that proved to be chemically stable.[51] Both ^{123}I and ^{131}I were used.

Follow-up animal distribution in tumor bearing rodents with the ^{131}I-bleomycin indicated a 12.1 tumor to blood and a 36.8 tumor to muscle ratio at twenty-four hours. Due to these encouraging results ^{123}I was used for scintigraphic comparison in patients. The researchers concluded that ^{123}I-bleomycin was rapidly distributed and cleared from body and that chemically, biologically and physically it was the most suitable radiolabeled bleomycin complex used thus far for tumor imaging.[52]

III. Radioiodination Techniques

The concept of using radiolabeled compounds, not only for metabolic and physiological studies, but also for the non-invasive techniques of anatomical definition, have gained widespread use in recent years. radioimmunoassay not only allows very precise measurement of the endogenous concentrations of natural hormones or proteins, but also the blood levels of exogenously supplied drugs or polypeptides. Most of these compounds make use of ring or side chain labeling using the β-emitters ^{14}C or ^{3}H. This involves liquid scintillation procedures which are sometimes long, require expensive fluor materials and extensive correction factors. Recently the use of weak gamma emitters

(e.g. ^{125}I) have become available in assay procedures. The ease of sample handling makes this a desirable technique. The need for methods and procedures were then sought to quickly and efficiently label proteins and polypeptides in particular. Of course with the advent of ^{123}I becoming more available these methods could then be utilized for the preparation of excellent imaging agents using the scintigraphic detection methods available today. The importance of iodination lies in the fact that it is a chemical or covalent bonding, which is stable, and not a chelate or complex formation.

A review of the commonly used methods of iodination of proteins or polypeptides follows.

The basic entity generally labeled in these procedures is the tyrosine residue. The mechanism involves an electrophilic substitution of the ortho hydrogen in the phenolate anion of tyrosine.[53] The iodine must be in the +1 oxidation state. As iodine is only sparingly soluble in aqueous solution (1.1 mM/l at 20°C) iodide, which forms soluble complexes, is added. A look at the reactions of iodine with solvents is necessary before an in depth look at the tyrosine labeling procedures are scrutinized.[54]

Equilibria of Iodine with Solvents

$$I_3^- \rightleftharpoons I_2 + I^- \qquad 1$$

$$I_2 + H_2O \rightleftharpoons H_2OI^+ + I^- \qquad 2$$

$$I_2 + OH^- \rightleftharpoons IOH + I^- \qquad 3$$

$$HOI \rightleftharpoons H^+ + OI^- \qquad 4$$

$$3OI^- \longrightarrow OI_3^- \qquad 5$$

$$I_2 + RNH_2 \longrightarrow RNHI + H^+ + I^- \qquad 6$$

Reaction 1. In the presence of iodide ion, molecular iodine is almost completely in the tri-iodide form.

Reaction 2. Indicates the ionization of iodine to yield the active species H_2OI^+. Since another iodide is produced this species rate of reaction must vary inversely as the square of the iodide concentration.

Reaction 3. Shows the hydrolysis of molecular iodine to hypoiodous acid and iodide.

Reaction 4. Shows the ionization of hypoiodous acid.

Reaction 5. Indicates an adverse sequel to ionization and iodination, the irreversible formation of iodate. This also sets an upper practical limit for iodination at a pH 10.

Reaction 6. Exhibits a competing reversible reaction involving amino groups. These reactions serve to depress the rate of reaction without altering the outcome.[54]

In 1947 Painter and Soper[55] studied the iodination of phenol using molecular iodine in water, and in the presence of buffers, and found the kinetics of this uncatalyzed reaction to be in agreement with the two reactions (2 and 3).

$$[C_6H_5OH][HOI]$$

$$[C_6H_5O^-][H_2OI^+]$$

In the second case no distinction is possible between the H_2OI^+ and I^+ (aq). The ambiguity arises because the proton can be either on the phenoxide ion or the hypoiodous acid.

A differentiation can be shown between the two iodinating species when aniline is used as the aromatic substrate.[53] Aniline has the same kinetic characteristics as phenol and the two possibilities again arise.

$[C_6H_5NH_3^+][HOI]$

$[C_6H_5NH_2][H_2OI^+]$

The first of these can be eliminated on chemical grounds: the anilinium ion is meta-directing whereas only ortho and para substituted iodination products were noted.[55] The inverse dependence on the square of the iodide ion concentration, noted above, has also been found in iodination in other aniline derivatives and imidazole. Therefore, the following scheme of mechanisms represents the iodination of aromatic (Ar) compounds.[53]

$$I_2 + H_2O \rightleftharpoons H_2OI^+ + I^-$$

$$H_2OI^+ + ArH \rightleftharpoons ArHI^+ + H_2O$$

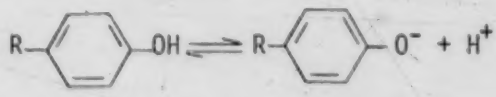
$$ArHI^+ \longrightarrow ArI + H^+$$

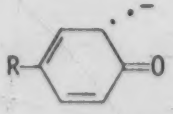
or

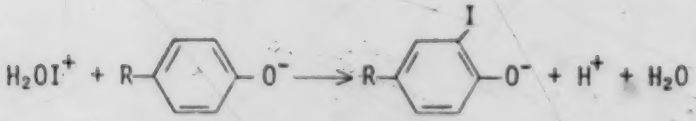
$$ArH + I_2 \longrightarrow ARHI^+ + I^-$$

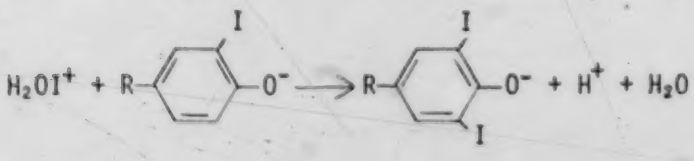
$$ArHI^+ \longrightarrow ArI + H^+$$

The principal reactions with proteins can then be summarized below. The equilibrium is generally far to the right making them essentially irreversible.[54]



The phenolate anion exists also in the quinoid form 





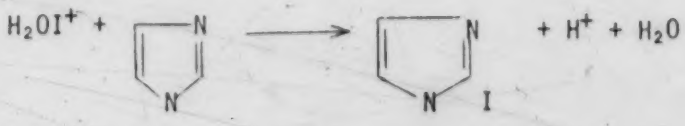
The following reactions can also occur in protein or polypeptidic iodinations.[54]

$$H_2OI^+ + RSH \longrightarrow RSI + H^+ + H_2O$$

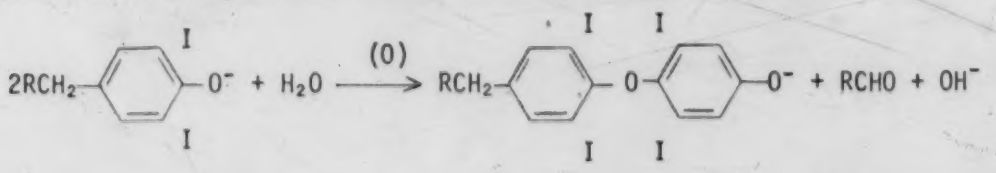
$$RSI + RSH \longrightarrow R\text{-}S\text{-}S\text{-}R + H^+ + I^-$$

The reactions of the sulfhydryl groups always occur much more rapidly than the phenolate reaction but they do not result in stable bonding of iodine and generally results in the immediate release of iodide.[56]

The following reactions can also occur when excess iodine is introduced into tyrosine-containing proteins that also contain histidine entities.[54]



In the latter stages of iodination of tyrosine, in the presence of excess iodine, thyroxine formation has also been observed.[57]



Although there is some controversy over the nature of the iodinating species, whether it is H_2OI^+ or I^+ (aq), they cannot be distinguished on kinetic grounds. It was found that isotope effects are the rule in iodination but not in bromination or chlorination.[53] The almost invariable participation of C-H bond-breaking in the rate determining step of iodination has been considered to be in agreement with the role of the base in buffer catalyses as assisting in breaking

the bond. It would appear therefore that iodinations are base catalyzed.[53]

A more detailed mechanism will follow on the iodination of imidazole residues. The iodinated derivatives of histidine have been prepared, containing either a C-I or N-I bond.[58] The carbon-iodine bond is the only stable one, however. Aside from tyrosine the only other amino-acid that is likely to be iodinated under normal conditions and with any degree of stability, is histidine. Tryptophan may be destroyed. The tyrosyl entities usually iodinate more readily but the difference is not extreme.

Many methods have been devised, for the iodinations of polypeptidic molecules. These methods include KIO_3 oxidation utilized by Stabb[59] and Utige[60] for the labeling of glucagon and growth hormone, nitrite oxidation used by Yalow[61] to radioiodinate insulin, persulfate oxidations to label albumin with ^{131}I utilized by Gilmore[62] and McFarlane has labeled plasma proteins using hydrogen peroxide as the oxidizing agent.[63]

Probably the methods utilized the most and certainly producing the most desirable products are the following four; the iodine monochloride method, the chloramine T method, radioiodination by enzymatic procedures, and an electrolytic technique.

A. Iodine Monochloride Method of Radioiodination

This procedure has been used successfully by a number of workers since its introduction by McFarlane in 1958.[64]

To incorporate radioiodine into proteins, this element must be converted into the positive iodine state as present in hypoiodous acid. This may be accomplished by oxidation or by isotopic exchange with non-radioactive hypoiodous acid or a substance that produces HOI on hydrolysis. e.g. I_2 or ICl. Only one atom in I_2 can acquire this positive character but in ICl all the iodine is available for iodination.

In McFarlane's[64] method, $Na^{131}I$ is added to an HCl-NaCl solution of ICl and brought to a pH 8.5 with a glycine-NaOH buffer. This mixture is injected into the protein solution, at a pH no higher than 9.5. The inorganic iodine is then removed by anion exchange or dialysis. The radioactive iodine could be added to the iodine monochloride before or after conversion to the hypoiodide state. In the best yields of 60-80% of the radioactivity bound to the protein, the glycine buffer was injected into the ^{131}ICl solution just prior to mixing with the protein.

Helmkamp's publication showed a modified version of this work.[65] He pointed out that when a buffer of basic pH is added to ICl, the reactions of its formation are reversed. The ICl_2^- hydrolyzes to Cl^- and HOI. The latter will rapidly decompose to I_2 and iodate and the iodine will in turn be transformed to iodide and more iodate. This process increases with the temperature and basicity of the buffer. This disproportionation can ultimately remove one-third of the total iodine and lead to inefficient labeling. This can be represented as follows:[65]

$$ICl_2^- + OH^- \rightleftharpoons 2Cl^- + HOI$$

$$5HOI + OH^- \rightleftharpoons 2I_2 + IO_3^- + 3H_2O$$

$$2I_2 + 2OH^- \rightleftharpoons 2HOI + 2I^-$$

$$3HOI + 3OH^- \longrightarrow 2I^- + IO_3^- + 3H_2O$$

This loss of efficiency can be minimized by adding the $Na^{131}I$, which is usually in alkaline media to the buffered protein solution and then adding the iodine monochloride with rapid and thorough mixing. The labeling of γ-globulin was increased to a consistent 81-86%. Helmkamp found the isotopic method to be more advantageous than oxidation of the radioactive iodide by iodate, especially at low levels of activity.[15] When, however, protein is iodinated with ^{131}ICl produced by the oxidation of the $Na^{131}I$, all the radioiodine becomes available for iodination. This procedure was useful when high specific activities or "over iodinations" were carried out. Helmkamp attained a 500 mCi/mg labeling of insulin using this method.[66]

Ceska et al.[67] has made even further modifications, one of which is the addition of sodium thiosulfate to stop the reaction and a dilution of his labeled γ-E globulin with 5% human serum albumin and 2% potassium iodide to reduce self radiolysis of the protein.

Ceska has also produced a mathematical model of the labeling of proteins with iodine monochloride.[68] The theoretical values were compared to experimental data and the results compared favourably. The amounts of ^{125}I iodide and the influence on ^{125}ICl formation were studied in depth.

An enlightening article by Hung et al[69] appeared in 1973 on the iodine monochloride reactions of aqueous solutions of L-tyrosine, L-histidine and L-histidine peptides.

At pH 4 L-tyrosine iodinated more rapidly than L-histidine but at pH 7.4 they iodinated at approximately the same rate. It was also shown that conformation of various peptides, or the surrounding amino acids, played a very important role in the catalytic influence, and subsequent iodination, of the imidazole ring of the histidine on ICl hydrolysis.[69]

B. Chloramine T Method

Chloramine T is the sodium salt of N-monochloro-p-toluene sulfonamide. It is a mild oxidizing agent and in methods described by Hunter and Greenword[70,71] a simple and rapid method was introduced for the preparation of ^{131}I-labeled human growth hormone at relatively high levels of specific activity (300 μCi/μg). They found approximately a 70% transfer of the ^{131}I to the protein using low amounts of carrier free radioiodine and small quantities of the protein. The degree of chemical substitution is minimal (0.5 to 1.0 atoms of iodine molecule^{-1}) and there was no apparent degradation of the molecule, as it proved to be immunologically identical with unlabeled hormone. This property deteriorated of course, as either higher specific activities or chemical substitution was attained.

The actual procedure consisted of reacting the various components in a rubber-capped vial in which 2 mCi of ^{131}I was shipped.[71] To this was added 0.025 ml of 0.5M phosphate buffer (pH 7.5), 5 μg of human growth hormone followed by 100 μg of freshly prepared chloramine T each in 0.025 ml of the phosphate buffer. The contents were briefly mixed, then 0.1 ml of a 2.4 mg/ml solution of sodium metabisulphite

in buffer was added, which acted as a reducing agent to halt the iodination. The residual iodide was then diluted with .2 ml of 10 mg/ml potassium iodide solution and the total reaction mixture was eluted through a Sephadex G-50 to separate the protein-bound fraction from the unbound iodide. The labeling efficiency was found to be time dependent and the amount of chloramine T used was critical.[71] This procedure caused denaturation of some proteins, however, particularly if high specific activity iodine was used.

A modified approach was introduced by McConahey and Dixon[72] to minimize denaturation by using lower chloramine T concentrations and longer reaction times. Fifty different proteins were iodinated using from 50 µg to 1000 µg of chloramine T with anywhere from 40 to 90% labeling depending on the protein. Varying amounts of oxidizing agent were necessary since the number of reducing groups in each protein which could interfere with iodination varied considerably.

Using these gentler methods Lamoureux et al.[73] labeled bovin encephalitogenic polypeptide to study its immunological properties. The biological activity was found to remain intact and on hydrolysis of this protein, it was noted that histidine as well as tyrosine had been labeled.

A comparative study on the properties of plasma lipo-proteins iodinated by the iodine monochloride and the chloramine T method has been carried out by Sundaram and his group.[74] They concluded that no one method was superior and the behaviour of the lipo-proteins was unaltered and identical regardless of the procedure. The iodine

monochloride method, however, proved to be somewhat easier and gave more reproducible results.

C. Enzymatic Methods of Radioiodination

Due to the ever-present danger of protein denaturation using the chloramine T or iodine monochloride methods of iodination, a gentle means of high specific activity iodination was sought. Enzymatic iodinations have been known since 1944, when Keston,[75] using crude mixtures containing various peroxidases and peroxide, or peroxide generating systems, produced iodinated tyrosine. The process involves the oxidation of iodide to iodine enzymatically.

The peroxidases, a widely distributed group of enzymes, are found in fungi, bacteria, all mammals and higher plants. Most reviews suggest that a common reaction mechanism exists for oxidations catalyzed by all peroxidases and that all peroxidases participate in the same types of reactions.[76] Hemoproteins and copper proteins will also exhibit peroxidase activity, but not to the same specificity and these have been termed pseudoperoxidases.[77] Four true peroxidases have been isolated and purified in mammalian tissue: the familiar thyroid peroxidase, myeloperoxidase present in white cells, glutathione peroxidase which is widely distributed, and lactoperoxidase which is localized in the mammary, salivary and lacrimal glands.[77]

Marcholonis was able to trace-label immunoglobulins and other serum proteins using lactoperoxide, hydrogen peroxide and iodide.[78] Gamma G-immunoglobulin was labeled to a specific activity of 5 μCi/μg with no apparent denaturation. He concluded the iodination was taking

place on the tyrosyl residues and the degree of labeling differed in various proteins probably due to conformational changes. Lactoperoxidase iodinated much more readily than horseradish peroxidase, an enzyme of plant origin.[78] The radioiodination reaction was carried out at room temperature and consisted of adding in rapid succession, the radioactive iodine-125, 250 µg of polypeptide or protein, 1.25 µg lactoperoxidase and 1 µl 8.8 mM hydrogen peroxide. The reaction was terminated with the addition of either 0.5 ml of 5 mM cysteine or 5 mM 2-mercaptoethanol.[78]

Thorell and Johansson[79] have successfully labeled a number of polypeptides including insulin, human growth hormone, human follicle stimulating hormones, human luteinizing hormone and thyrotropin, to high specific activities using a modification of this method.[76] Levels of iodine substitution ranged from 0.5 to 1.5 atoms per mole or protein and activities of 300 µCi/µg were realized. The reaction was successful at pH levels of 3 to 8 and 80% binding was obtained with no appreciable loss in the immunological behaviour observed.

Bayse, Michaels and Morrison made a complete study into the peroxidase catalyzed iodination of tyrosine and tyrosine containing peptides.[80,81] This group found that only lactoperoxidase catalyzed the direct iodination of tyrosine, while horseradish peroxidase catalyzed the oxidation of iodide and the iodination was a result of the reaction between the I_2 and the phenolic group.[81] There was also a clear indication of stereospecificity in the two peroxidases. Lactoperoxidases iodinated the L-isomer more readily than the D-isomer while the opposite was true for the horseradish peroxidase.[81]

The basic method of Marcholonis[78] has been utilized on other proteins as well. Prolactin has been iodinated by this procedure and the reaction parameters studied indicated that by decreasing the hydrogen peroxide, and increasing the enzyme and the reaction time, excellent binding was obtained with high specific activity. The biological activity remained intact.[82]

In screening all the common methods of radioiodination of fibrinogen Krohn and Welch have chosen the lactoperoxidase method.[83] They found this procedure was non-degrading to the protein and, although a small yield of iodohistidine was noted, it appeared more specific for tyrosine. The stability of the product was more pronounced than that of the other methods at comparable specific activities.

D. Electrolytic Method of Radioiodination

The electrolytic process as originally described by Rosa[8] was found to have several advantages over the conventional chemical oxidation or isotope exchange methods. These advantages included: no need of using oxidizing agents, which provided certain control over the liberation of iodine, and the possibility of using dilute solutions of carrier iodine (KI) which allowed the amounts of iodine introduced into the protein molecule to be reduced for the same specific activity. This method, of course, removed the direct contact of polypeptides with oxidizing agents and thus reduced the danger of oxidizing other protein constituents such as the breaking of disulfide bridges.[8]

Rosa's technique involved the electrolysis of a $K^{131}I$ solution to produce elemental iodine and thus label human fibrinogen. This process was carried out at neutrality in the presence of physiological saline. A constant current was fed to a electrolytic cell consisting of a glass beaker with a cylindrical platinum sheet coating the inside as the anode; the cathode was a fine platinum wire in a glass tube support, whose lower end is closed by a cellophane dialysis membrane of 48Å pore size. The reaction mixture was comprised of 100 mg of fibrinogen in normal saline, and 2.5×10^{-4}M KI in 10 ml labeled at tracer levels with ^{131}I. The cathodic compartment was filled with the same KI solution.[8]

The yield of iodinated product remained constant at currents between 100 μA and 500 μA, but for higher current values the iodine loss was significant and degradation products were noticed. Most of the iodinations were carried out at 300 μA for thirty minutes with a 73-77% yield at specific activities of 50 μCi/mg. There was no denaturation of the fibrinogen observed.[8]

Subsequent experiments by this group using human albumin pointed to a more complete understanding of the constant current electrolysis iodination of polypeptides.[8] A 100% theoretical yield could be obtained with the anodic discharge of the iodide ions being the rate-limiting step in the whole process. It was found that up to ten atoms of iodine could be introduced into the albumin molecule using low current values with no apparent structural damage observed.

A platinum crucible was used in these experiments and a lower limit of carrier iodide was determined to be $.5 \times 10^{-4}$ M for

elemental iodine to be generated at a 100% current yield. The pH was normally adjusted to 7 and the temperature was controlled to about 8° to 10°C.[8]

Rosa et al.[8] found that prolonged electrolysis had no effect on the protein but did not enhance the bonding once a maximum had been reached. The entire process was reproducible and even predictable due to the reoxidation of the reduced iodine at the anode. The iodination proceeded even in the presence of reducing agents, although a longer electrolysis time was then required.

Katz and Bonorris repeated the original work of Rosa, except that they used higher currents and a larger anodic surface.[84] These workers obtained yields in the 80 to 90% range if the current was adjusted to 1 to 2 mA. The biological studies on their iodinated albumin and alpha amylase showed it to be of superior quality when compared to those obtained by the ICl method.

Insulin was successfully iodinated in 1969 by Pennisi and Rosa using modifications of their first cell and using much lower current values.[85] They introduced an average of six atoms of iodine per mole of insulin with no degradation and attained yields of 90%. In the case of insulin, it was found that currents in excess of 100 μA actually gave less iodinated product than expected and it was assumed that the iodine incorporation rate into insulin was slower than other proteins and certainly slower than the formation rate. It was also noted that at higher iodination rates diiodotyrosine and iodohistidine were detected as well as the monoiodotyrosine.[85] These findings confirmed the work of Coveli and Wolf[86] who had previously detected

substantial amounts of iodinated histidine in bovine insulin when labeled with extensive amounts of iodine. A micro-electrolytic procedure was developed in 1971 by Donabedian and his associates permitting the iodination of minute amounts of hormones with ^{125}I to high specific activity.[87] Microgram quantities of porcine insulin, human growth hormone and thyroid-stimulating hormone were electrolytically labeled at one iodine atom per molecule using a minute teflon cell 4 mm in diameter and 5 mm deep. The anode was a piece of platinum foil at the base and the cathode, which also revolved to accomplish mixing, was a fine platinum wire. The electrolysis was conducted at 22°C with a constant current of 5 μA for forty-five minutes using a ratio of one atom of iodine from $Na^{125}I$ to one molecule of hormone. The products retained their immune reactivity and when used in radioimmunoassays optimum displacement of the unlabeled hormone resulted.[87]

Parathyroid hormone is very sensitive to oxidants and thus the usual chemical methods resulted in degradation of the molecule. An electrolytic procedure utilizing Rosa's technique[8] was conducted by Sammon et al[88] in 1973. It was found that 50% of the biological activity had disappeared from the product and this was assumed to be due to oxidation of the parathyroid hormone. When the iodinated hormone was treated with mild reducing agents a partial restoration of the biological activity was found. Accordingly voltage-time relationships were adjusted to minimize oxidation due to electrolysis. It was concluded that if the voltage was kept below 800 mV and the electrolysis run for thirty minutes an undamaged highly labeled

hormone resulted. The yield reached 97% radio-iodine labels on a fully intact molecule.[88]

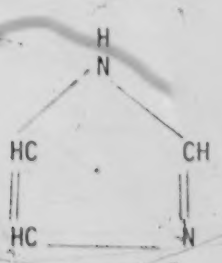
IV. Histidine Chemistry

Various workers in the routine iodination of proteins have noticed a higher degree of iodination than would be expected if only the tyrosyl entities were being labeled. This is due to the concomitant iodination of the imidazole ring of histidine. Li[89] measured the rate of histidine iodination and he has shown that under normal iodinating conditions it is probably the only other amino acid likely to be substituted, although tryptophan may be destroyed.

The imidazole ring is a tautomeric structure which can exist in fifteen resonance forms.[90] It is very stable chemically and exhibits a typical aromatic behaviour. It contains a sextet of electrons, but in contrast to a benzene system where each methene group contributes on π electron, the ring partners in a heterocyclic compound do not make equal contributions to the resonance system. The methene groups and the tertiary nitrogen contribute one electron each in contrast to the imino group which is the source of two electrons. The pyrrole or imino nitrogen is acidic in character, by virtue of the π electron distribution, which gives it a positive charge and thus facilitates proton release.[90]

The tertiary nitrogen, which is characteristic of the azoles, imparts the basic properties to the imidazole and are strikingly similar to those of the nitrogen in pyridine. This function has been designated as the pyridine nitrogen.

The presence of the pyrrole and pyridine nitrogens explains the amphoteric quality of the structure. A deactivating influence could be expected toward electrophilic reagents, caused by the pyridine nitrogen, but this is offset by the electron releasing properties of the pyrrole nitrogen.[90]



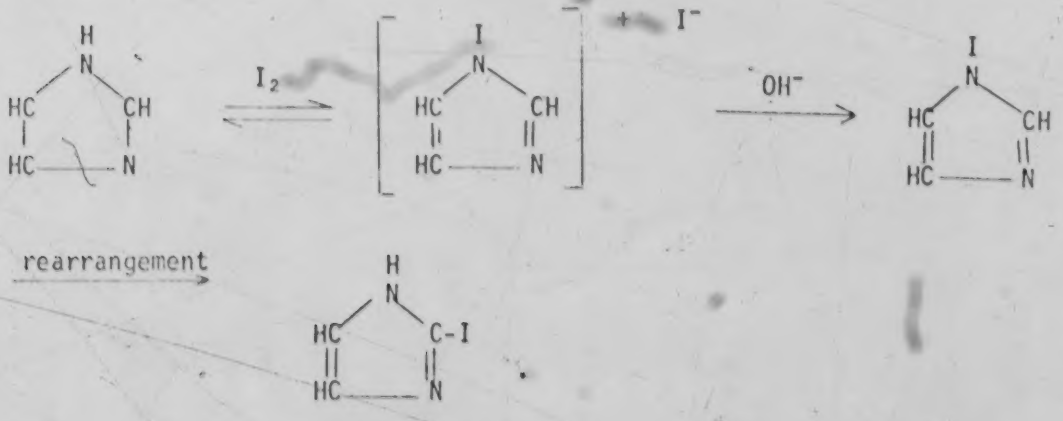
The imidazole containing amino acid, histidine, cannot be synthesized by most mammals and therefore L-histidine is considered an essential amino acid. This Zwitterion has an isoelectric point of pH 7.2 and plays an important role in hemoglobin formation. The histidine unit in the globin molecule forms the bond with the hemin molecule through the imidazole nitrogen and the iron atom.[90]

A. Halogenation

Some of the original work carried out on the iodohistidine and its derivatives brought forth a number of interesting points. Bruning[58] pointed out that the iodination of histidine is contrary to the bromination or chlorination of this residue. Mono-iodination occurred in the two carbon position preferentially while bromination or chlorination occurred at the four or five position. It was assumed, therefore, that iodination was not a simple electrophilic substitution on

the carbon and involves a complex formation mechanism. Bruning suggested a primary N-bonded intermediate on the imino group with a subsequent base catalyzed rearrangement.[58]

There are a number of requirements for this successful iodine bond according to Bruning.[58] The imino nitrogen has to be free, an alkaline medium is preferred. In the presence of an oxidant the reaction proceeds more favourably and it would appear that atomic iodine is the attacking species on the imidazolium anion.



The iodohistidine product has an increase in the pseudoacidic character of the molecule due to the introduction of electronegative substituents. The carbon-iodine bond is much more resistant on the carbon 2 than in the four or five position.[90]

Although iodohistidine have been suspected in proteins after iodination procedures were carried out *in vitro* Roche *et al.*[91] was the first to demonstrate their presence in globin and thyroblobulin. From 1966-1969 Covelli and Wolf demonstrated the formation of iodohistidyl residues in lysozymes,[92] ribonuclease A[93] and insulin.[86]

These proteins were all iodinated at low temperature and at a pH of 8.5 using Tris-HCl buffer. They also suggested that the presence of phosphate ion had a suppressive effect on the histidine iodination of ribonuclease A and thus the reason for using Tris buffers.[93] This group summed up their work by outlining the various factors that control the iodination of the imidazole entity in various proteins.[94] In total there were thirteen various polypeptides, of hormonal or enzymatic function, tested. The following conclusions were reached.

1. Fewer histidyl residues were iodinated than tyrosyl residues at pH 8.5 but the fraction increases with the pH.
2. The mechanism proceeds by a direct attack on the imidazolium anion contrary to Bruning's[90] nitrogen-iodine intermediate theory. This is supported by the finding the sterically hindered 2-t-butyl imidazole is iodinated.
3. The degree of iodination of the histidyl residues on any particular protein molecule is quite different from another individual molecule due to conformational or electrostatic influence.
4. The histidine species is known to be an active site of binding, and failure to iodinate could be due to the binding of small molecules, hydrogen binding or ligand binding (such as Zn^{++}).[94]

More recent publications have exhibited the labeling of L-histidine, at neutral pH, using the iodine-monochloride method.[69]

A comparison of the various methods of iodination on free L-histidine, L-tyrosine and various peptide combinations was carried out by Krohn et al.[83] in his work with lactoperoxidase. Labeling was accomplished with all techniques and histidine residues were

iodinated quite substantially using chloramine T, but to a lesser degree enzymatically. There was ample evidence of iodohistidine present in radioiodinated fibrinogen.

EXPERIMENTAL

I. Materials

A. Animals

Young male white mice of the Swiss IRC strain weighing 20-25 grams were used throughout the study. The mice were housed in a relatively stress-free environment in groups of no more than six per cage with food and water ad libitum.

B. Chemicals

All chemicals were of A.C.S. specifications and triple distilled water was used throughout the experiments.

The following chemicals were purchased from Fisher Scientific Co. Ltd., Fair Lawn, New Jersey.

1) Potassium Iodide (KI) Molecular weight 166.01
   Lot number 724913
2) Sodium chloride U.S.P. (NaCl) Molecular weight 58.44
   Lot number 781914
3) Ammonium Acetate (CH_3COONH_4) Molecular weight 77.08
   Lot number 731022
4) Tham ^{R} - Tris-Hydroxymethyl aminomethane $C_4H_{11}NO_3$
   Lot number 726519
5) Other manufacturers
   a. Methanol (99.5% CH_3OH) was supplied by McArthur Chemical Co. Ltd., Montreal.
   b. Ethanol (95% C_2H_5OH) McArthur Chemical Co. Ltd., Montreal.

6) Acids

a. HCl concentrate lot number 705101 supplied by Fisher Scientific Co. Ltd. This was diluted in 500 ml to give a working solution of .2 N HCl.

7) Buffer Solution

Buffer solutions were made up according to the Buffer Solution table contained in Geigy Tables.

The buffer range was from pH 7.2 to pH 9.0.

8) Bleomycin complex - Blenoxane ^{R} - Bristol Laboratories of Syracuse, New York. The lyophilized contents of the vial contains 15 units of bleomycin complex and in actual weight was 8.4 mg. A typical batch contains 53% A_2, 12.2% A_1, 6.6% A_2', 1.7% A_5, 4.4% B_1, 19.9% B_2 and 2.2% B_4.

9) Bleomycin pure A_2 standard was supplied by the Nippo Kayaku Co. of Tokyo, Japan and was a generous gift from Bristol Laboratories Ltd., Syracuse, New York.

C. Chromatographic Material

1) Silica Gel for column adsorption chromatography was purchased from B.D.H. Chemicals Ltd. in Poole, England, lot number 1906960. It was 60-120 Mesh.

2) Kieslegel ^{R} for thin layer chromatography was supplied by Camag of Switzerland with an ultra-violet indicator added. A binder of calcium sulfate ($CaSO_4 \cdot 2H_2O$, McArthur Chemical Co. Ltd., Montreal) was added.

3) Sephadex G-10 [R] This is a bead formed dextran gel prepared by cross-linking selected dextran fractions with epichlorhydrin and supplied by Pharmacia of Dorval, Quebec. The dry particle diameter ranges from 40 to 120 microns and the gel has a water regain value of one.

Sephadex G-10 has a fractionation range or molecular exclusion limit of 700-1000 molecular weight for polypeptide or globular proteins.

4) Eastman Chromagram Sheets #6061 are supplied by the Eastman Kodak Co. of Rochester, New York. These consist of 20 x 20 cm sheets of fine silica gel on a mylar (polyethylene terephthalate) support. The silica is approximately 100 microns in thickness.

5) Gelman ITLC SG [R] strips, lot number 20201, were supplied by the Gelman Instrument Co. of Ann Arbor, Michigan. These are a glass microfiber impregnated with a fine silica gel. They were in 5 x 20 cm strips.

6) Dowex 1 x 4 Anion Exchange Resin

Purchased from BioRad Laboratories of Richmond, California, this weak anion exchange resin consists of beads of a polystyrene matrix with quaternary functional groups in the chloride form. It is the large pore size of a 100-200 mesh.

D. Radioisotopes

1) Iodine 125 - This was supplied from ICN Pharmaceutical of Irvine, California in the form of $Na^{125}I$ carrier free. A specific activity of 540 mCi/ml was received in .02 ml of .1N NaOH. This isotope was

immediately diluted to .2 ml with .1N NaOH and assayed for radiochemical and radionuclidic impurities. The solution was then frozen until used.

2) Technetium 99m - This nuclide was supplied from a New England Nuclear ^{99}Mo-^{99m}Tc generator Lot number 7522-2-175 of Boston, Massachusetts. The eluate was assayed for radiochemical and radionuclidic purity and the yield of ^{99m}Tc was measured with an Isotope Calibrator model 34-C05 (Nuclear Associates, Westbury, N.Y.).

II. Methods

A. Chromatographic Techniques

1) Bleomycin A_2 Isolation with Silica Gel Column

The separation of the various sub-fractions of bleomycin on thin layer chromatography using a 10% ammonium acetate:methanol (1:1) solvent is well documented and serves as a method of identification.[12] The A_2 sub-fraction being the most abundant and apparently the most specific and least toxic has an R_f of .4. This value is determined by measuring the distance the material in question migrates on the medium compared to the distance the solvent front moves from a point of origin. This can be represented by the formula:

$$R_f = \frac{\text{distance from origin to spot centre}}{\text{distance to solvent front from origin}}$$

This separation depends on the polarity of the compound in question and the polarity of the mobile phase (solvent) of the system as well as the adsorbant attraction between the stationary phase (silica) and the compound.

Blenoxane ^{R}, the commercially available copper-free bleomycin complex, contains only seven sub-fractions of which the A_2 makes up about 53% of the total weight. On the basis of the wide separation of the R_f value of the A_2 from the rest of the complex, it could be possible to isolate it from the other components with silica gel column chromatography.

Silica gel was triple washed alternately in IN HCl and distilled water to remove foreign organic material. The gel was then washed with absolute methanol and left to hydrate in the solvent system of 10% ammonium acetate:methanol (1:1) for twenty-four hours.

A K9/60 (.9 x 60 cm) acrylic column (Pharmacia, Dorval, Quebec) was packed with a slurry of the washed silica gel using a slow continuous pour. A Vortex mixer was used to vibrate the column base quite vigorously during the pouring procedure. This method insured very tight compaction of the gel with a minimum of fractures, air pockets or bubbles and channeling. The above technique also provided a flow rate of never more than 0.1 ml minute^{-1} on numerous separations. This tight packing was necessary for absolute separation.

A solution of bleomycin complex (Blenoxane ^{R}) was made containing 8.4 mg/.5 ml. An aliquot of this solution (.2 ml) was applied to the silica column.

The column effluent was monitored by passing it through a 1 cm quartz flow cell in a Beckman Model DB ultraviolet spectrophotometer (Beckman Instruments Ltd., Fulerton, California), set at a wavelength of 254 nm. A matching 1 cm quartz flow cell filled with solvent was used as the reference. Results of absorbance were recorded automatically on a 10" Beckman Recorder (Beckman Instruments Ltd., Fulerton, California).

2) Sephadex G-10 Purification

The fractions of eluate from the silica column were collected and concentrated in a warm water bath, under a stream of air, down to a volume of approximately 0.2 ml.

A K9/15 (0.9 x 15 cm) acrylic column (Pharmacia, Dorval, Quebec) was packed using a continuous pour method, using slight column vibration, with a Sephadex G-10 slurry. This gel slurry had been previously hydrated for twenty-four hours in triple-distilled water. The various concentrated fractions from the silica gel column were applied to this column and developed using distilled water as the solvent. Again the eluates were passed through a 1 cm quartz flow cell and absorbance monitored using the Beckman DB spectrophotometer at 254 nm. This method served to effectively de-salt the fractions and also gave a profile of the various components present in each fraction.

An aqueous solution of Bleomycin A_2 standard containing 5 mg/ml was also made and .2 ml of this material was also applied to the Sephadex G-10 column as a reference.

The fractions from the column were concentrated using a warm water bath and an air stream to approximately 0.1 ml and freeze dried in the initial procedures. This method was very time-consuming and resulted in the loss of product. Subsequent purifications involved the concentration of the eluate to near dryness as described above. The residue was then dissolved in 2 ml of methanol and concentrated to .5 ml with heat. When 4 ml of pre-cooled 95% ethanol was added, a fine white crystalline precipitate formed. The supernatant was removed with a pipette and the crystals then dried in a dessicator overnight. These residues were then re-dissolved in very small volumes of distilled water (.025 ml) and identified as to composition by chromatographic analysis.

3) Thin Layer Chromatographic (tlc) Assays

a. Non-radioactive techniques on tlc (glass)

Glass plates 2 mm thick (5 x 20 cm) were coated to a uniform thickness of .5 mm using a Quick-Fit Plate Spreader (Quick-Fit Instrument Co. Ltd., England). The slurry was prepared with 60 gm of Kieslegel ^{R}, with uv indicator, 3 gm calcium sulfate and 120 ml of distilled water. The plates were air-dryed and stored in a desiccator until required.

Bleomycin complex (Blenoxane ^{R}) was dissolved in distilled water to make solutions of 8.4 mg/ml and bleomycin A_2 standard was dissolved in water to make a solution of 5 mg/ml. Both these dilutions were spotted on the tlc plates

to confirm R_f values reported in the literature and also as standards for identification of unknown bleomycin complex solutions. The spots were kept to 2 mm in diameter and dried with a gentle air stream. Approximately .015 ml was used for each spot.

The plates were developed in a conventional glass chromatography tank using freshly prepared solvent which was allowed to equilibrate in the tank for twenty-four hours. The plates were developed to a distance of 10 cm and the spots detected with a short wave ultraviolet lamp.

b. Radioactive or Radiochemical Assays on tlc (Glass)

An identical procedure for the preparation spotting and development of the plates was followed with the radiolabeled bleomycin. This chromatographic technique was used to not only determine which sub-fraction of the complex was being preferentially iodinated but also as an accurate determination of radiochemical purity of the reaction product. This method also served to distinguish any differences in migrational behaviour of the molecule after labeling, as compared to the unlabeled material, and would also exhibit the presence of degradation products if any.

In the treatment of the radioactive plate assays, ten 1 cm segments were marked off and each section of gel scraped into individual BioVials (Beckman Instruments Limited, Fulerton, California). The activity of each vial was then determined using the Beckman Biogamma (Beckman Instruments

Limited, Fulerton, California). Window settings on this instruments were automatically set, using the ^{125}I module supplied with the spectrometer, to count the twin energy peaks of ^{125}I with an efficiency of 84%. This value was determined using a known specific activity standard. Due to the simplicity of operation, high counting efficiency and ease of sample handling, this instrument was used on all thin layer procedures assays throughout the project.

All calculations for radiochemical purity on tlc systems were based on the total plate count method. The total activity on the plate was considered to be 100% of the activity spotted on the plate and all segments were then interpreted as percentages of that total. This method accounts for all residual activity on the chromatogram and yields a more accurate value for the bound and unbound isotope.

c. Eastman Chromagrams for Radiochemical Purity

The 20 x 20 cm sheets were cut into strips approximately 1.5 cm in width. The strips were spotted as previously described and developed, using 95% ethanol as the solvent, in 2.5 x 15 cm glass test tubes. The mylar-backed strips were held in place in the solvent using split-corks and developed to 10 cm. After air-drying, ten equal segments were then cut at 1 cm intervals, placed in Bio Vials ^{R}, and the segment activity determined using the Beckman Biogamma. All assays using this system for radiochemical purity were run in

triplicate at various time intervals, during, and at the termination of iodination procedures.

d. Gelman ITLC ^{R} -SG Assays for Radiochemical Purity

The 5 x 20 cm strips of glass microfiber were cut to fit the Seprachrom Chamber (Gelman Instruments, Ann Arbor, Michigan). They were spotted with approximately 0.005 ml of compound and developed in 95% ethanol until the solvent front reached the pre-dawn line of the chamber. This method was very rapid and was complete in seven minutes compared to seventy-five minutes for the Eastman chromograms. The strips were air-dryed, cut into five equal segments, placed in Bio Vials and the activity counted in the Beckman Biogamma. Again all radiochemical determinations were run in triplicate at different time intervals.

4) Dowex 1 x 4 Ion Exchange Resin

This material was hydrated and stored until use in .1N HCl to retain it in the chloride form. It was used in the terminal step of the iodination procedures to remove any free iodide from the reaction mixture.

Five ml syringe barrels were used as columns and fitted glass frit discs were placed above and below the Dowex 1 x 4 bead bed. This maintained the bed bead, which was approximately 2 mm thick, in a stationary position during rinsing or backwashing operations. The syringes were then clamped firmly to immobilize them in an upright position and the anion exchange resin was washed to neutrality using distilled water. Just prior to use, all excess

water was forced from the bead bed with air pressure and then the reaction mixture of bound and unbound iodine was applied to the resin. Once the liquid was applied and migrated into the resin the syringe was capped at the lower end and exchange allowed to take place. After ten minutes the bound compound was forced from the bead bed with air pressure to the top of the column. A wash solution of .5 ml of distilled water was then applied and moved back and forth through the beads, using a syringe plunger and finally expelled with air pressure and pooled with the bound compound.

B. Quality Control of Radioisotopes

1) Iodine-125

a. Radionuclidic Purity

The energy spectrum of the stock $Na^{125}I$ solution was determined using a NaI (Tl) detector. The spectrum was stored in a multichannel analyzer (Northern Scientific, Middleton, Wisconsin) and recorded on the x-y plotter.

The peaks observed in figure 2 can be attributed to the 35 KEV and the 60 KEV sum peaks, characteristic of ^{125}I. No radionuclidic impurities were apparent.

b. Radiochemical Purity

The radioiodine in the form of $Na^{125}I$ was chromatographed on the Eastman chromograms, Gelman ITLC and the standard tlc (glass support) to ascertain radiochemical impurities using the described solvent systems.

These methods also served to later identify the iodide or iodate activity that may be present in reaction mixtures.

2) Technetium-99m

a. Radionuclidic Purity

The tests for radionuclidic purity on the NEN ^{99m}Tc-generator were carried out to determine if any ^{99}Mo was present.[95] The energy of the ^{99m}Tc radiation (140 KEV) is much less than the energetic gamma rays of ^{99}Mo (740 KEV, 780 KEV). By adequately shielding the generator eluate sample and counting in the 600 to 900 KEV range the presence of ^{99}Mo may be detected. A simulated ^{99}Mo source consisting of ^{137}Co was used as a reference. The reference and eluate were counted in the same geometry and total ^{99}Mo present was calculated using the following formula.

$$\mu Ci\ ^{99}Mo\ (total) = \frac{\mu Ci\ of\ reference \times cpm\ of\ eluate}{net\ cpm\ of\ reference}$$

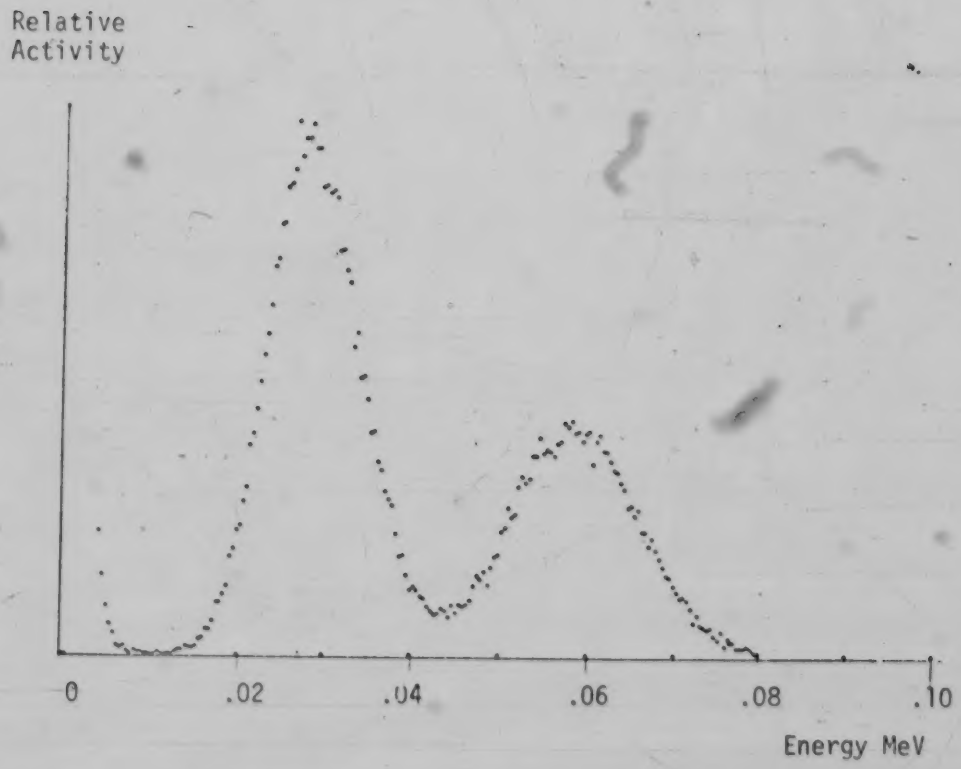
When this answer is divided by the millicuries of ^{99m}Tc the total ^{99}Mo impurity is determined. The acceptable limit is 1 μCi ^{99}Mo/mCi ^{99m}Tc. The pertechnetate fell within this limit.

b. Radiochemical Purity

The sodium pertechnetate eluted off the generator was spotted on Gelman ITLC and developed using .9% NaCl according to the Procedure Manual of the Gelman Instrument Co.[96] In this system the technetium in the +7 or unreduced state, migrates as the pertechnetate with the solvent front. The

Figure 2

Radionuclidic Purity of $Na^{125}I$



^{125}I Energy Spectrum Obtained from Sodium Iodide Scintillation Detector

reduced technetium in the +4 state will not migrate in this solvent and remains at the origin as an impurity. The eluate should contain 95% or more unreduced pertechnetate to be considered radiochemically acceptable.

C. Bleomycin Labeling Procedures

1) Electrolytic Radioiodination

a. Electrolytic Cell

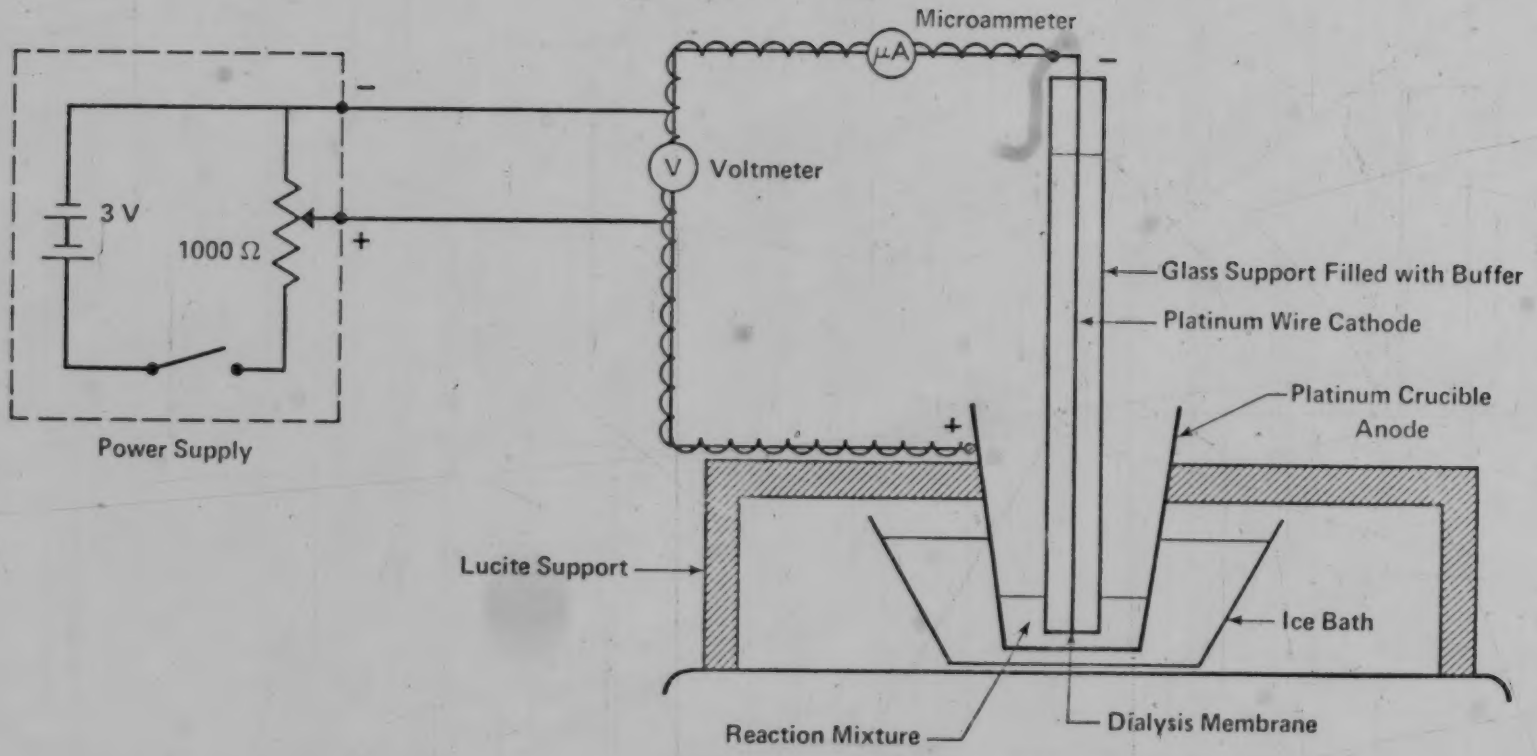
The basic cell used in the electrolytic procedure is essentially the same as that of Pennisi and Rosa's[85] second cell. It consists of a platinum crucible 25 mm in diameter by 25 mm in depth in which the reaction takes place and serves as the anode. The cathode is a thin platinum wire .8 mm in diameter, supported in a glass tube and separated from the reaction mixture in the anodic compartment by a cellophane dialysis membrane with a pore size of 48 Å. The cell is supported by a Lucite stand positioned on an ice bath container and magnetic stirring device.

b. Electronics

The voltage was supplied by two 1.5 volt type "D" dry cell batteries attached to a constant voltage supply. This was connected directly to the anode and cathode terminals of the cell, as was a voltmeter (Armaco Multi Range DC Voltmeter, Model SM331, Armaco, Japan) to measure the potential difference between the poles. A microammeter (Armaco

ELECTROLYTIC IODINATION CELL

Figure 3



Multi Range DC Microammeter, Model SM301, Armaco, Japan) was connected to the cathode to measure the current through the cell. The electrolytic cell and the associated electronics are illustrated in figure 3.

c. Parameters Studied

Knowing that it is possible to iodinate the imidazole ring under certain conditions,[94] it was necessary to study the following parameters under which optimum labeling could be obtained:

(1) pH - Various pH conditions had to be optimized and of course, the closer to the physiological pH the better. Since some researchers had experienced difficulty using phosphate containing compounds in iodinating histidine,[94] Tris-Cl buffers were used throughout.

(2) Temperature - The effects of temperature upon the electrolytic radioiodination procedure was determined in the range of 0°C to 25°C.

(3) Time - Since the iodination of histidine is a much slower reaction than that of tyrosine and there is only one residue in bleomycin that will form a stable covalent bond, the time of exposure to the electrolysis would therefore be necessarily different than that used in tyrosine labeling procedures upon which this method was based.

(4) Voltage - current relationship. Optimum voltage was determined by measurement of the amount of iodate formed during electrolysis.

(5) Peptide - Carrier iodide ratio. This ratio was varied to some extent, however the basic concept of the procedure dictates that a 1:1 ratio of KI:bleomycin should be ideal.

d. General Procedure for Electrolytic Radioiodination of Bleomycin

All the platinum and glass components were washed thoroughly in a chromic acid bath and rinsed with distilled water. Freshly prepared, precooled aqueous solutions of carrier iodide, .1N NaCl and Tris buffer were used throughout. Once the parameters had been established for a particular reaction a blank run was carried out using all the ingredients except the radioiodine. The reaction mixture consisted of carrier iodide, bleomycin A_2 or bleomycin complex in a Tris Cl buffer at the desired pH which is .1N in NaCl. This reaction was run to determine if molecular degradation of the bleomycin was occurring during electrolysis. The various R_f values of the product were compared to an unreacted bleomycin standard using the aforementioned procedure utilizing conventional tlc. All reaction mixtures were 1 ml in total volume and the reactions were conducted in a well ventilated fume hood.

A blank run was also conducted using ^{125}I but in the absence of bleomycin to determine if there were components of the supporting system which were acquiring a radiolabel during electrolysis.

For a true run all the components were mixed thoroughly in the cell for ten minutes without electrolysis. This zero time analysis would give an indication of any labeling that may occur spontaneously. A 0.01 ml aliquot was then removed and spotted on tlc, Eastman chromogram and Gelman ITLC. After the current has been supplied to the cell, .010 ml aliquots were removed at time intervals of thirty minutes, sixty minutes, 120 minutes and 180 minutes and spotted for radiochemical analysis according to methods described. This procedure gave quantitative results of the progress of the reaction. A qualitative assay was performed using the following simple detection technique, at the same time intervals.

Aliquots of .025 ml were removed from the reaction mixture and applied to small Sephadex G-10 columns. These columns were prepared using 10 ml syringe barrels into which approximately 3 gm of Sephadex G-10 slurry is poured. Using distilled water as the solvent, the eluate was monitored by passage through a fine canula tube positioned in front of an adequately shielded NaI (TI) crystal detector which was connected to an electronics system composed of a Canberra Model 1417 B Spectroscopy Amplifier, a Canberra Model 1481 L Lin/Log Ratemeter, a Canberra Model 456 High Voltage Supply and a Canberra Model 1437 Timer S.C.A. (Abtec Engineering, Ottawa) All results were recorded on a Beckman 10" Recorder (Beckman Instruments, Fulerton, California).

As the bleomycin has a molecular weight and size which is excluded from the gel pores, it is eluted off the column first, at the column, void volume, while the iodide or iodate is retarded slightly. This method gives a general indication of the radioactivity in each fraction and thus the progress of the electrolytic labeling may be assessed.

At the termination of each electrolytic process the reaction mixture was passed through the Dowex 1 x 4 resin column to remove any free iodide, as previously described. The resultant eluate was again chromatographed to determine radiochemical purity.

2) Technetium-99m Labeling of Bleomycin

The technetium labeling, by means of stannous pyrophosphate, was carried out following the method recently described by Bardy et al.[47]

A stannous pyrophosphate kit that had been previously prepared and frozen at the Dr. W.W. Cross Cancer Institute was used as a source of stannous pyrophosphate. The kit consists of 2.1 mg of stannous chloride, and 59.7 mg of tetrasodium pyrophosphate in a volume of 1 ml. Sterile pertechnetate was eluted from the ^{99m}Tc NEN generator. The bleomycin complex (4.1 mg) was dissolved in 0.5 ml of pertechnetate and .076 ml of the stannous pyrophosphate was added to supply a total of 4.2 µg of tin. The solution was mixed adequately for five minutes in a shielded beaker and the product analyzed chromatographically by the three

methods of thin layer chromatography as described to determine radiochemical purity.

D. Stability Tests on ^{125}I-iodobleomycin

After termination of various electrolytic procedures the product was stored at 5°C and at 22°C, in the original vial, for time periods up to thirty days. Daily aliquots were removed for one week and then weekly, for the remainder of the month. These aliquots were chromatographed on the various tlc systems to determine if any dehalogenation was occurring.

The product was also placed in a water bath at 100°C and left for one hour to assess thermal stability of the radiolabel.

The possibility exists that the bleomycin may chelate or electrostatically bond the iodine rather than form a covalent bond on the imidazole ring. A series of experiments were conducted to challenge the iodine-bleomycin bond using an aqueous solution containing copper ions. The molarity of the Cu^{++} solutions was kept equal to the molarity of the iodobleomycin. If the iodine was simply chelated by the bleomycin then the copper ions should displace the iodide due to the preferential chelation of copper by bleomycin. A solution of copper sulfate was added to the labeled bleomycin at neutral pH, and vigorously mixed for thirty minutes. The resultant solution was spotted on the standard tlc systems and assayed.

E. Animal Studies

1) Preparation of Tumor Model

Mice bearing intraperitoneal Ehrlich ascites tumor cells, previously innoculated, were a gift from McEachern Cancer Laboratory, University of Alberta. These animals were housed and otherwise treated in an identical manner as the normal healthy animals. The intraperitoneal tumor cells were allowed to incubate in vivo for approximately one week, at which time the animals were sacrificed by ether asphyxiation. The abdominal wall was incised and the ascites fluid was withdrawn from the abdominal cavity into sterile syringes. An aliquot of this fluid was pro-essed using a Coulter Counter and estimated to contain approximately 10^7 cells per ml.

Approximately 0.1 ml of this fluid was injected subcutaneously into the right femoral area of healthy young male mice. After five to seven days a solid non-mobile tumor of one to two cm had developed in all animals. Ampicillin was administered in the rodents' drinking water (.25 gm/200 ml) to rule out the possibility of bacterial infections at the site of injection.

2) Animal Studies of Radioiodinated Bleomycins

In the studies of the tissue distribution of the labeled bleomycin products, all animals were injected, via the tail vein, with .05 or .1 ml of specific activity ranging from 25 μCi/mg to 150 μCi/mg of either bleomycin A_2 or complex. Forty-two animals, without tumors, were injected, via tail vein with .05 ml (75 μCi/mg)

of iodobleomycin complex. At time intervals of one hour, two hours, three hours, four hours, six hours, twelve hours, and twenty-four hours, groups of six animals were serially sacrificed. The thoracic cavity was immediately opened and 0.1 ml of whole blood was withdrawn with a syringe by direct cardiac puncture. Representative tissue samples were excised from the lung, kidney, liver, spleen, testes, and leg muscle. All tissue samples were rinsed with distilled water and blotted dry to remove excess blood and accurately weighed.

All samples were then placed in clean glass gamma counting vials. The syringe used for blood collection was triple rinsed with normal saline and the washes pooled with the blood sample. The samples were then assayed for ^{125}I activity using the Searle Model 1195 (Nuclear Chicago, Des Plains, Illinois) gamma spectrometer.

In all the animal studies the total radioactivity administered was determined by counting an aliquot of the original preparation and calculating the equivalence of the volume injected. The activity in the tissue samples was sufficiently high to allow one minute counts on all samples.

A similar procedure was followed using iodobleomycin A_2. A specific activity of 50 μCi/mg was used in all forty-two animals of this series.

In the tumor model tissue distribution experiments the groups of six animals were serially sacrificed at the same time intervals. Included in the tissue specimens excised were three transverse

sections of the tumor. In all tumor animal series conducted, the distribution of radioiodinated bleomycin complex, ^{125}I-bleomycin A_2 and ^{99m}Tc-bleomycin was studied.

In all distribution studies the remainder of the carcass was assayed by placing the remains in a plastic container and determining the radioactivity using twin probe NaI (TI) crystals, suitably shielded, connected to a Canberra Model 3000 CI High Voltage Supply, an Ortex Model 486 Amplifier pulse-height analyzer and a Canberra Model 1772 Counter timer.

3) Excretion Studies

Three normal mice were injected via the tail vein with ^{125}I-iodobleomycin complex of various specific activities, which had been assayed and predetermined. The animals were kept in separate cages and whole-body activity was determined at zero time, two hours, four hours, twelve hours, one day, two days, three days and four days after injection.

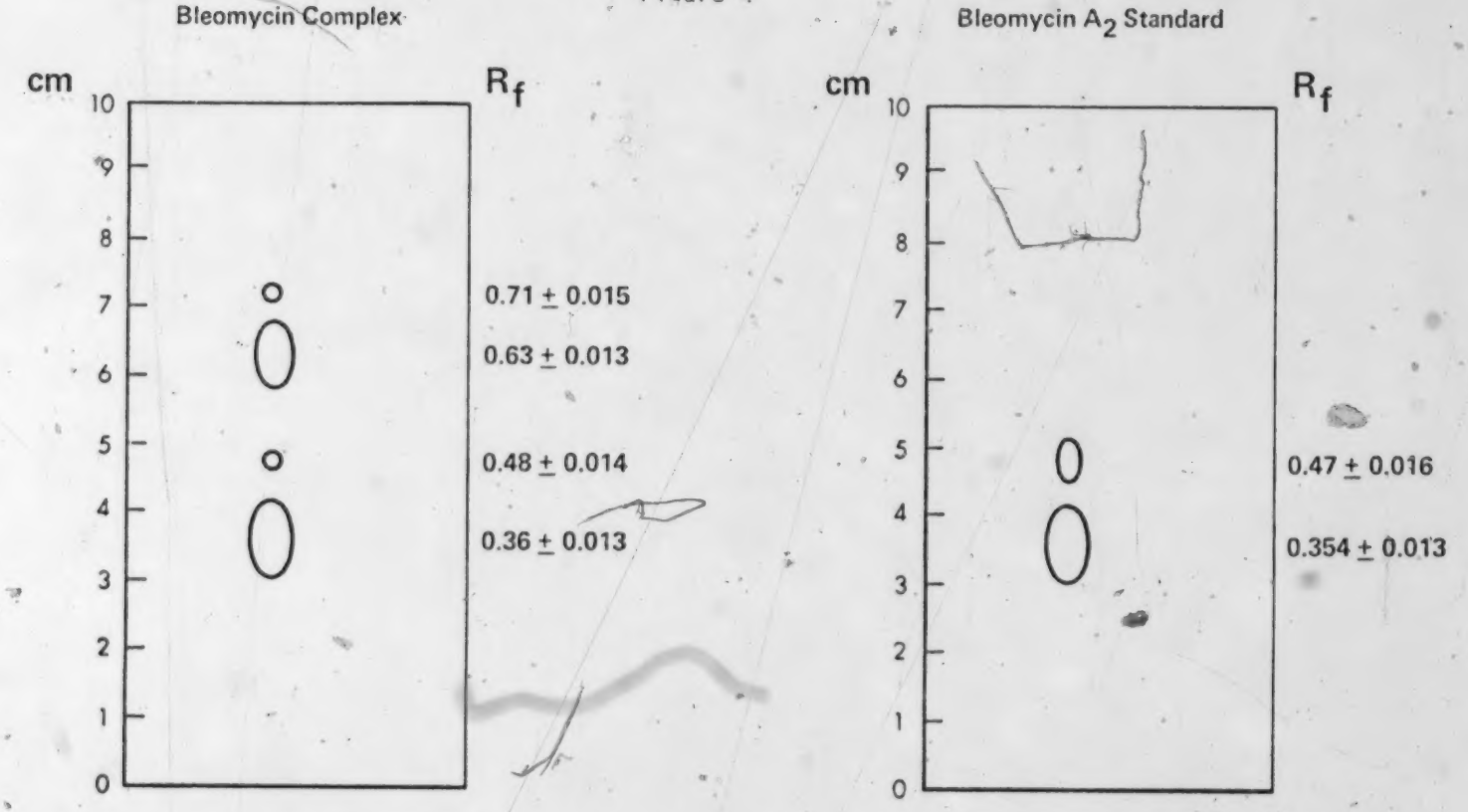
RESULTS AND DISCUSSION

I. Characteristics of the Bleomycin Complex on T.L.C.

An 8.4 mg/ml solution of bleomycin complex (Blenoxane) in distilled water was made up as a working solution for confirmation of the migrational behaviour of the compound. Thin layer chromatography was carried out by spotting .015 ml of this material on the glass-backed medium and developing in 10% ammonium acetate:methanol (1:1) in a closed chromatography tank at room temperature. After development to 10 cm, air drying the plates and utilizing a short-wave ultraviolet light for spot detection, four distinct areas on the chromatograms were visible. The largest had a mean R_f .36 ± .01. A small very diffuse spot had an R_f of .48 ± .01, a larger spot was at an R_f of .63 ± .013 and a small separate spot at R_f of .71 ± .02. These R_f values and standard deviations were the results of eight separate experiments and are illustrated in figure 4.

The various spots were isolated and scraped from the plates and placed in 5 ml pyrex beakers. One ml of distilled water was added to each and mixed for ten minutes to elute the compound from the silica gel. The gel was allowed to settle and the supernatant was withdrawn and concentrated to .05 ml using a hot water bath and air stream. The resultant concentrates were spotted on a strip of Whatman #1 filter paper and air dried. The strip was then dipped in .02% phenanthraquinone in absolute ethanol and 10% sodium hydroxide in 60% ethanol (1:1). Serial dilution studies showed that this indicator solution will detect the guanidino group of arginine in concentrations as low as ten parts per million.

Figure 4



Characteristics of Bleomycin Complex (Blenoxane) and Pure A_2 on TLC (a)(b)

(a) Developed with 10% ammonium acetate:methanol (1:1) at 20°C.
(b) Visualized with a short-wave uv lamp.

After adequately drying, the strip was viewed using a short wave ultraviolet light under which arginine-containing compounds flouresce. The spot at R_f of .63 ± .013 was positive with a slight flourescense seen at the spot of R_f .71 ± .015. Since the guanidino group is present only in the B complex sub-fractions, the B sub-fractions were present in the higher R_f value spots but absent in the lower spots. These values are in agreement with the published R_f values for the components of bleomycin complex (Blenoxane) which are A_2 - R_f .4; A_5 - R_f .51; B_2 - .68; A_1, and B_1 - .74.[12]

Another series of plates were developed in an identical manner with the exception that the plates were first activated by heating at 100°C in an oven for one hour. There were no changes in the migration behaviour of the complex and the R_f values were identical.

The bleomycin A_2 standard was spotted in an identical manner using .015 ml aliquots of the 5 mg/ml stock solution. After development a large spot was detected at R_f of .360 ± .012 with a small diffuse spot noted at R_f of 47 ± .016. It appeared that the A_2 standard actually contained a small percentage of a compound that corresponds to the A_5 sub-fraction chromatographically. The bleomycin complex also exhibited this spot at the same R_f value.

II. Silica Gel Column Separation of Bleomycin A_2

Due to evidence that the A_5 and A_2 sub-fractions of bleomycin were less toxic and more tumor specific than the other sub-fractions[4] it was desirable to separate these components from the rest of the

complex. As the A_2 fraction composes more than half the bleomycin complex, it would appear to be the most economical component to extract for recovery of workable quantities. The wide separation of the A_2 from the other sub-fractions on thin layer chromatograms in the solvent system described formed the basis for this procedure.

The washed silica gel slurry was poured into a K9/60 column and packed as described. The appropriate connections via canula tubing were made to the flow cell and the reference cell was filled with solvent. These were placed in the cell compartment in the Beckman spectrophotometer and solvent from a 250 ml reservoir was allowed to pass through the system. This served as a check for column fractures and leakages in the system. Clean calibrated glass 15 ml centrifuge tubes were used for fraction collecting. The machine was calibrated at this point with the reference cell and the solvent flowing, to 100% transmission or 0 absorbance at a wavelength setting of 254 nm.

A 0.2 ml aliquot of a 8.4 mg/.5 ml solution of bleomycin complex was applied to the top of the gel bed, care being taken not to disturb the gel, and the elution was begun with 10% ammonium acetate:methanol (1:1) at a flow rate of 0.1 ml/minute.

The Beckman recorder was set to run at .1 inches per minute on the log scale at 100 mV. A base line was established and the first fraction came off the column after an elution volume of 19.6 ml. Figure 5 illustrates graphically the elution profile. Three milliliter fractions were collected individually thereafter until elution was complete. After elution with a total of 42.3 ml the absorbance returned to zero, indicating an absence of bleomycin fractions, and

remained there for a further 6.2 ml. A second major absorbance peak was observed at an elution volume in excess of 50 ml and this peak was collected in 3 ml portions until the absorbance returned to zero after a total elution of approximately 70 ml. A similar separation was exhibited on subsequent experiments. The fifteen 3 ml fractions were then concentrated by evaporation at 40°C under a stream of air down to approximately .2 ml in the centrifuge tubes. Initially a freeze drying procedure for concentration was attempted, but proved to be unsuccessful due to the ammonium acetate present in the solution.

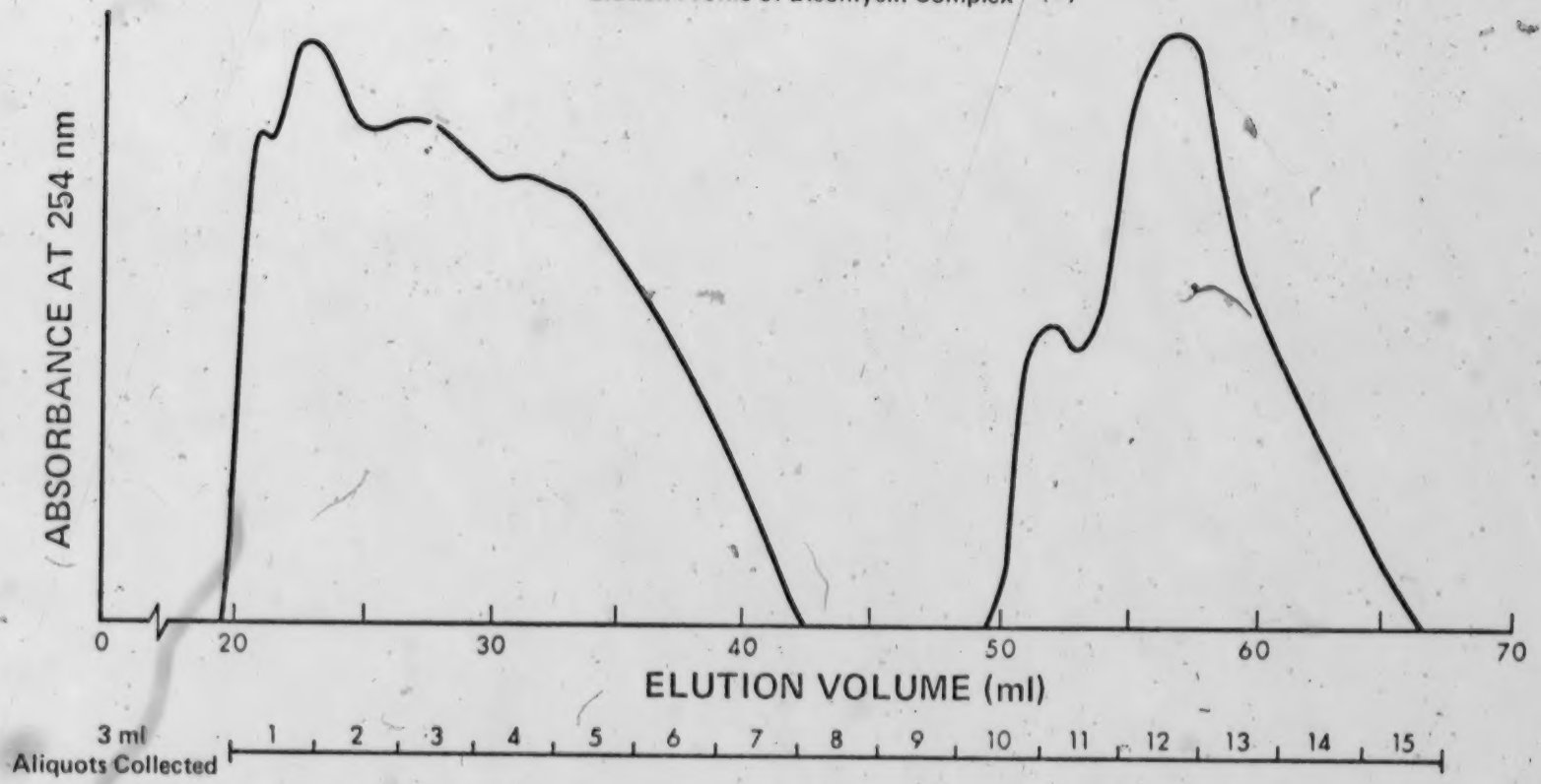
Aliquots of .025 ml of the fifteen individual concentrates were spotted on thin layer chromatography plates for identification and another .025 ml was spotted on Whatman #1 filter paper to determine the presence of the guanidino groups of the B sub-fractions. As illustrated in Table 2, the last peaks eluted off the column all gave negative arginine flourescent reactions indicating absence of B components. The R_f values of this peak corresponded to the published values of A_2 and A_5 sub-fractions when analyzed on tlc.

All fractions obtained from the large rather diffuse peak gave R_f values consistently above .65 and all gave positive arginine reactions both which are indicative of the B sub-fractions of the bleomycin complex. It was not possible to distinguish any A sub-fractions, particularly A_1 at these R_f values due to the close migrational patterns of the B sub-fractions which obscure the A components.

The absence of B sub-fractions and a positive identification of the A_2 sub-fraction, with minor concentrations of the A_5 components, in the last peak eluted, was indicative of a complete separation of the desirable sub-fraction from the commercially available bleomycin complex.

Figure 5

Elution Profile of Bleomycin Complex (a)



(a) Profile obtained by elution of bleomycin complex through silica gel column using 10% ammonium acetate:Methanol (1:1) at 20°C.

Table 2

R_f Values of Fractions Collected on Separation of Bleomycin Complex (Blenoxane) on Silica Gel, along with Arginine Test for B sub-fraction identifjcation. All values are the mean ± S.D. for six determination experiments

Fraction			Arginine Stain
1	.71 ± .011		+
2	.70 ± .014		+
3	.70 ± .012		+
4	.67 ± .012		+
4	.66 ± .015		+
5	.65 ± .016		+
6	.66 ± .008		+
7	.66 ± .016		+
8			-
9			-
10	.35 ± .016	.47 ± .020	-
11	.36 ± .012	.46 ± .012	-
12	.35 ± .018	.47 ± .014	-
13	.35 ± .012		-
14	.35 ± 0.18		-
15	.36 ± .011		-

III. Sephadex G-10 Purification and De-Salting Procedure of Bleomycin A_2 Sub-fraction

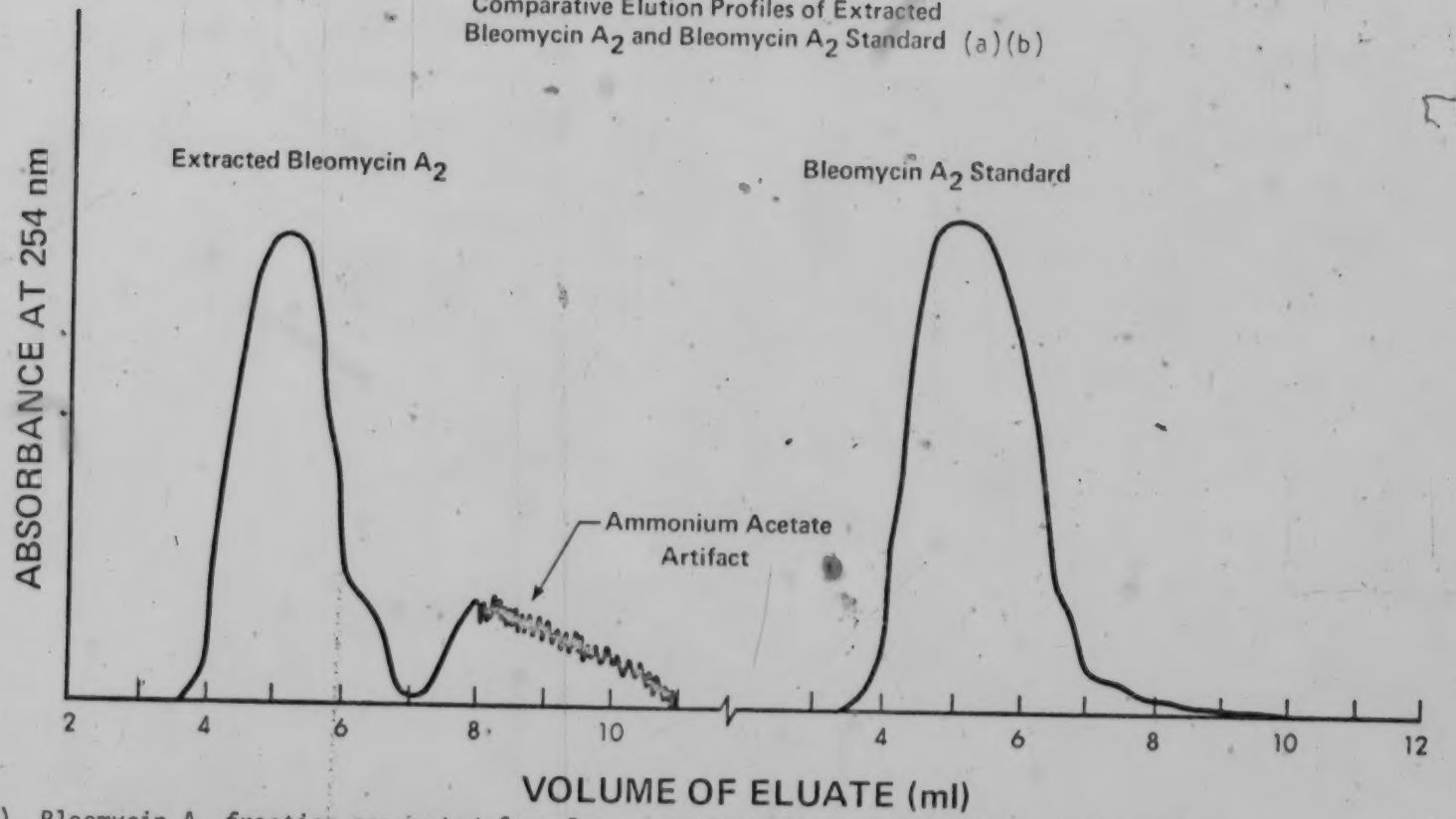
The K9/15 column was packed as described with a continuous pour method of a hydrated Sephadex G-10 gel. The column was connected via cannula tubing to the flow cell in the Beckman DB spectrophotometer with distilled water as the reference solution. Distilled water, supplied from a 250 ml reservoir, was eluted through the system. The instrument was adjusted to 100% transmission and zero absorbance at 254 nm and a base line established on the Beckman 10" recorder. The recorder was set at .5" per minute on the log scale at 100 mV. With the flow rate set at .3 ml per minute, the .2 ml fraction concentrates from the silica gel extraction were applied individually to the gel bed and eluted. The absorbance peak was noted at an elution volume of 3.9 ml, and fractions containing the complete absorption peak were collected in about a 3 ml volume of water. Figure 6 illustrates the elution profiles of the bleomycin A_2 fraction, which was isolated from the bleomycin complex by silica gel column chromatography, and that of the A_2 standard.

The main objective of this separation was to obtain bleomycin A_2 in as pure a form as possible. The 3 ml aliquots, numbered 10-15, from the silica gel extraction, were the only portions of the eluate confirmed to contain the A_2 sub-fraction on tlc, and thus were the only ones passed through the Sephadex G-10 for de-salting.

A comparative elution profile was also conducted under the same conditions using .2 ml of the 5 mg/ml bleomycin A_2 standard. On

Figure 6

Comparative Elution Profiles of Extracted Bleomycin A_2 and Bleomycin A_2 Standard (a)(b)



(a) Bleomycin A_2 fraction separated from Bleonoxane by silica gel column chromatography
(b) Profiles obtained by distilled water elution through Sephadex G-10 Columns.

elution both the standard and the separated bleomycin A_2 had very similar absorbance peaks. It can be noted from the profile graph that the retardation of the ammonium acetate on the gel insures adequate separation from the peptide product.

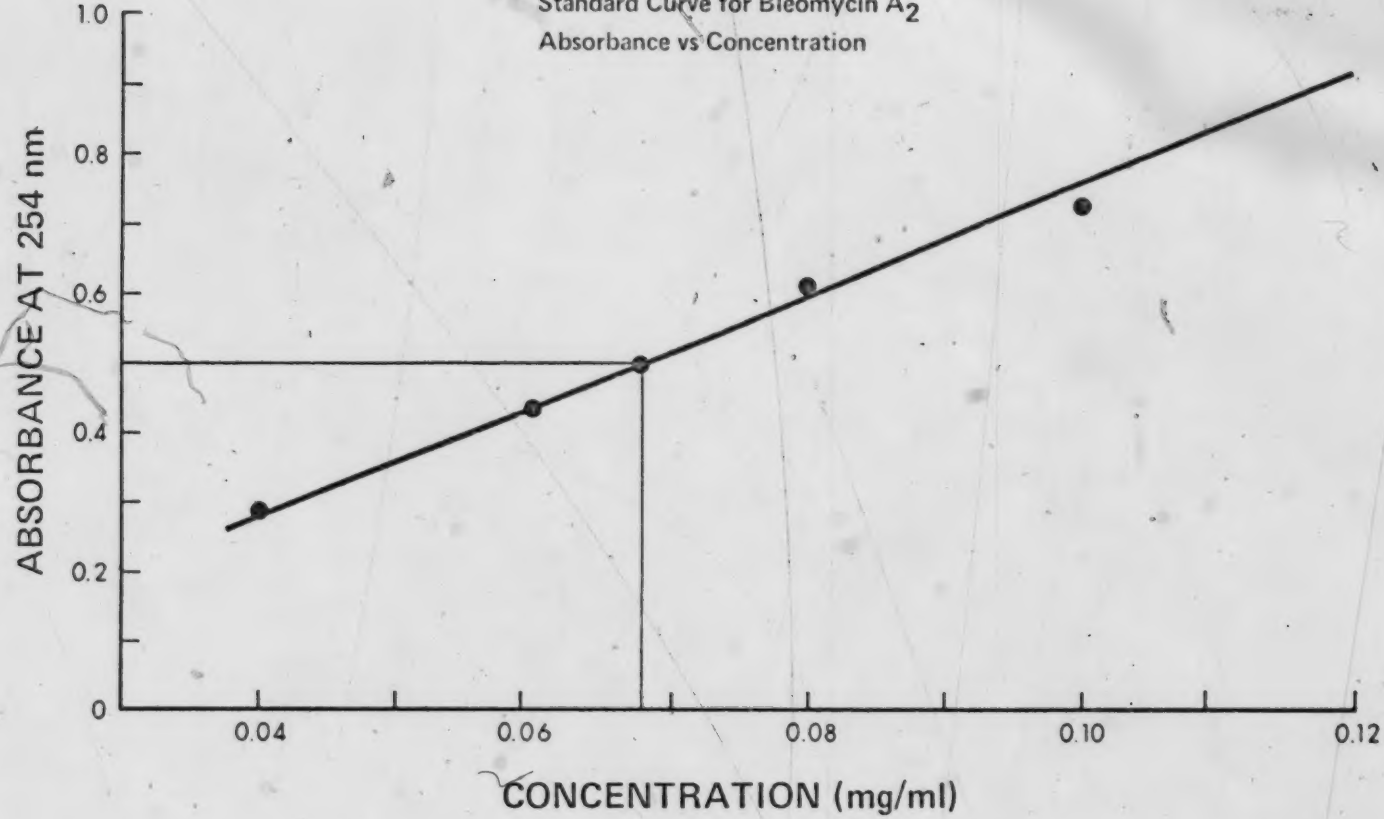
The five 3 ml eluates from the Sephadex G-10 de-salting procedure were then concentrated using a 40°C warm water bath under a gentle stream of air to a volume of approximately .5 ml each. The solutions were then pooled and a .025 ml aliquot was spotted on tlc to confirm the presence of bleomycin A_2. The R_f values substantiated the spots corresponding to A_2 and a trace of A_5 as exhibited on the original chromatographic analysis.

The pooled samples were then further concentrated to a volume of 1 ml and 3 ml of methanol added and then further concentrated to .5 ml. When 4 ml cold 95% ethanol was added to the concentrate a fine white crystalline precipitate was formed. The supernatant was withdrawn and the crystals allowed to dry in a desiccator. These crystals proved to be highly purified bleomycin A_2 with a trace amount of bleomycin A_5 and were used in the subsequent iodination and animal procedures.

To determine if this method was a practical procedure for bleomycin A_2 isolation from the commercially available bleomycin complex the mean yield of bleomycin A_2 from the various separation procedures was estimated. A standard curve, as shown in figure 7, using serial aqueous dilutions of the bleomycin A_2 standard versus the absorbance at 254 nm was constructed from the following data.

Figure 7

Standard Curve for Bleomycin A_2
Absorbance vs Concentration



Standard Curve for Bleomycin A_2 Standard

Absorbance at 254 nm	Conc (mg/ml)
0.92	0.120
0.73	0.100
0.61	0.080
0.44	0.060
0.36	0.050
0.29	0.040

The dry residue powder resulting from the silica gel separation followed by gel exclusion purification was dissolved in 10 ml of distilled water and the solution assayed spectrophotometrically.

The theoretical yield using .2 ml of the 8.4 mg/.5 ml solution would be 3.36 mg of total complex or 1.85 mg of bleomycin A_2 and A_5.

The results indicate a concentration of .066 mg/ml of A_2 or a yield of 37.5%. The numerous steps in this procedure undoubtedly accounted for a great portion of this loss. There was considerable loss on the columns due to adsorption both to the chromatographic gels and the glassware.

IV. Radiochemical Purity of Radioisotopes

A. Iodine 125

Aliquots of the carrier free radioiodine in the form of $Na^{125}I$ were spotted on the three thin layer chromatographic systems. This procedure was employed to determine the presence of any other chemical

form of the iodine and also to obtain a reference R_f value of free iodide for future analyses. The behaviour of this radionuclide was almost identical on the three systems using 95% ethanol as a solvent.

1) Thin layer chromatography (glass support silica gel media)

The main peak of radioactivity, after development with 95% ethanol to 10 cm in a conventional chromatography tank, was detected at an R_f value of .85-.9. This was determined by scraping individual 1 cm segments of the gel from the plates and assaying a suitably calibrated gamma spectrometer.

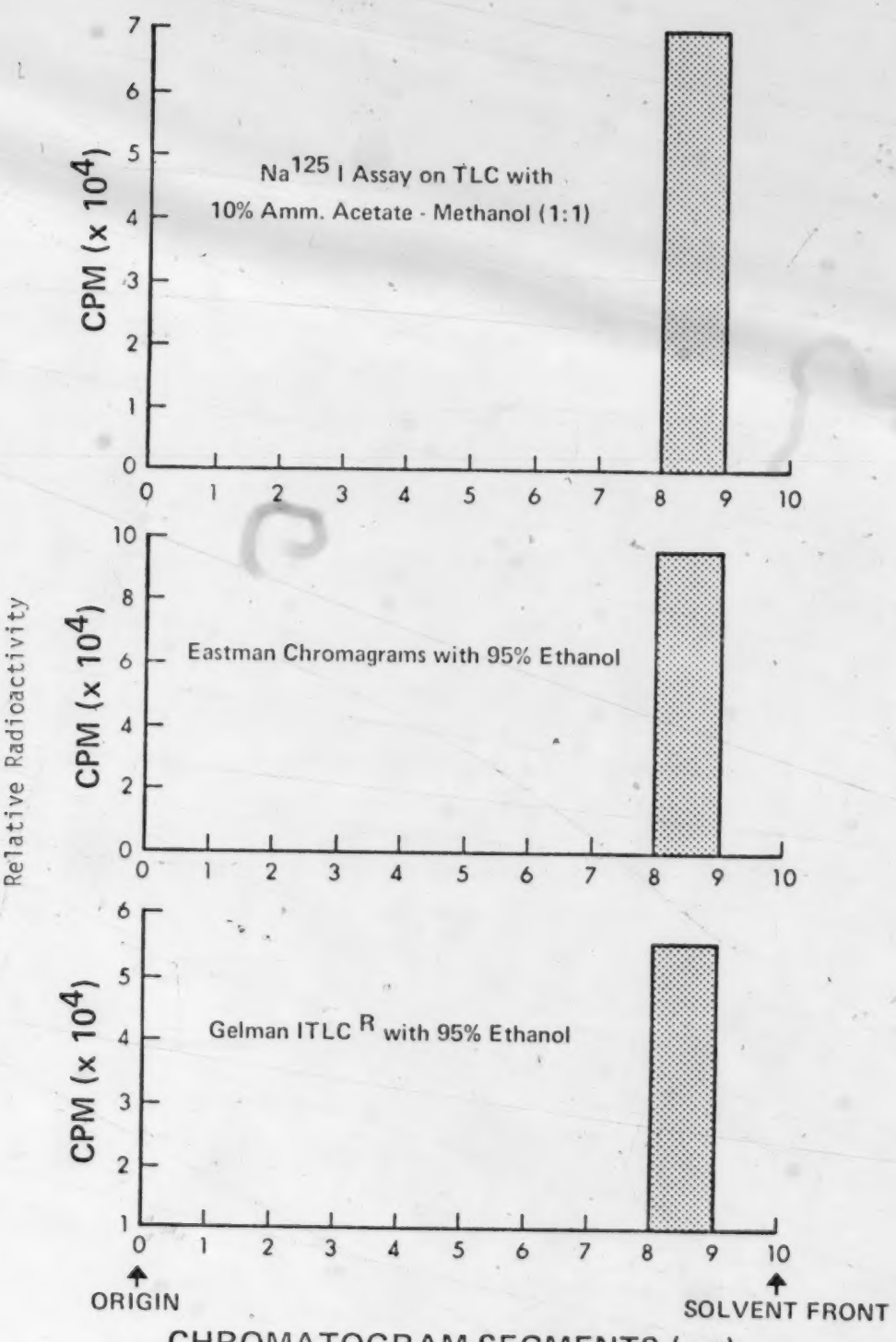
An identical R_f value of .9 was also exhibited when $Na^{125}I$ was developed on this same medium using the 10% ammonium acetate: methanol (1:1) solvent system. In both cases, more than 98.9% of the radioactivity was in this peak, with the remainder being distributed evenly over the plate as a residual radioactivity. Figure 8 is a representative histogram of these assays.

2) Eastman chromagrams

This assay method was found to be more rapid than the glass plate procedure and the radioiodide peak again migrated to an R_f of .85 in 95% ethanol. When exposed to ultraviolet light, the iodide could be visualized as a yellow spot. The position of this spot was confirmed by cutting the mylar backed strips into 1 cm segments and analyzing the radioactivity in each. Due to a thinner layer of silica on the support, the residual radioactivity on the strip was much lower and the iodide peak accounted for 99.7% of the total activity.

Figure 8

Characteristics of $Na^{125}I$ on TLC Systems



3) Gelman ITLC ^{R}

The small microfiber strips were spotted and developed in 95% ethanol in the Seprachrom Chamber ^{R}. Migration to the solvent front line was complete in only six minutes. Of the total radioactivity on the strips, 99.1% was retained at an R_f of .85. No other peaks were noted and the remaining activity was evenly distributed on the plate.

B. Technetium-99m

An aliquot of the sodium pertechnetate eluate from the ^{99m}Tc generator was spotted on the Gelman ITLC and developed using .9% NaCl as a solvent. Of the total radioactivity the remaining 96.4% was located at the solvent front with 3.6% of the ^{99m}Tc activity evenly distributed over the chromatogram. The pertechnetate eluate was above the acceptable level of 95% radiochemical purity.[96]

V. Bleomycin Labeling Techniques

A. Electrolytic Iodination

Prior to studying the various parameters necessary to yield optimum radioiodination of bleomycin, a preliminary labeling experiment was conducted using conditions adapted from other iodination procedures. Although evidence pertaining to the radioiodination of histidine by the electrolytic method is very sparse in the literature, there are, however, a number of documented factors that lead to acceptable labeling

levels in chemical iodinations of the imidazole ring.[94] By applying these guidelines arbitrarily a radioiodinated form of bleomycin was obtained but at a very low percentage yield. From this point the conditions under which the reaction took place could then be varied until optimum yields could be achieved.

Covelli and Wolff, in their work on histidyl residues of insulin[86] and later in a summary article on histidine iodinations of a number of proteins,[94] compared a number of factors leading to successful radioiodine labeling. They point out that one of the major factors of imidazole iodination is the conformation of the peptide and the accessability of the histidine entity. The histidyl moeity of bleomycin has been shown to actually project from the molecule and is stereochemically in a favorable position.[17] The presence of phosphate ion either in a buffer system or as a pyrophosphate or nucleotide will block or inhibit iodine incorporation.[93] For this reason Tris Cl buffers are the more desirable system to maintain pH. They also emphasize that divalent cations, such as Zn^{++}, may also block the binding site.[93] This has already been exhibited and noted with the bleomycin structure's chelating ability.

The substitution of iodine into the carbon-2 position of the heterocyclic ring requires a free imino proton and is base catalyzed.[58] These conditions can be met by carrying out all procedures at neutrality or in alkaline environment. Most iodinations of proteins appear to proceed more favourably at low temperature, probably due to the stabilizing effect on protein.[94] The incorporation of iodine was also noted at lower molar ratios of carrier iodide/peptide if the temperature was around 0°C.[93]

A complete range of iodide/protein molar ratios have been used from fourteen to one down to equimolar concentrations. The incorporation of iodine into histidine is slow and occurs much later than the labeling of the phenoxide anion of tyrosine.[93] If the molar ratios were increased much beyond 1:1 ratio, there was no substantial increase of incorporation into a peptide containing only histidine.[94] The rate limiting factor would seem to be the bonding to the ring and not the production of oxidized iodine. This would introduce an increased time element that is not prevalent to any extent in normal tyrosine iodine labeling.

In the electrolytic process a theoretical yield of 100% can be obtained due to the recycling of the iodide and its subsequent oxidation. Rosa and Penissi, utilizing a number of the previously mentioned parameters were able to attain encouraging yields of iodinated fibrinogen[8] and human serum albumin.[85] In these experiments it was noted, however, that high current values resulted in actual loss of iodine and molecular degradation occurred. Prolonged electrolysis at low current values appeared to have no ill effects on the polypeptides.[85] Subsequent iodination procedures using the electrolytic cell were conducted at 100 μA or less for longer time periods.[85] Donabedian et al.[87] confirmed this variation in Rosa's original method by successfully radioiodinating various hormones at current values of 5 μa for forty-five minutes.

Sammon and his associates, utilizing Rosa's technique, observed considerable oxidation of the parathyroid hormone they were attempting to iodinate.[88] By adjusting the voltage-time relationship they arrived

at the conclusion that to minimize molecular oxidation the voltage must be maintained at 800 mV or less.

Accumulating all these facts the following general parameters were used in the electrolytic cell.

All buffer solutions were Tris Cl and the pH was maintained from neutrality to 8.75. The solutions were pre-cooled prior to use and an attempt, using an ice bath, was made to maintain the temperature of the reaction at 0-5°C. The bleomycin was kept at constant molarity, dissolved in the buffer which contained .1M NaCl,[8] and varying molarities of carrier iodine were added.

The current values were kept at less than 100 μA and usually at 50 μA. The voltage was varied but usually kept at .8V or less.

A reaction mixture consisted of: 5×10^{-3}M bleomycin A_2 or complex, .1M Sodium Chloride, 50 μCi - 2 mCi of Na^{125}, and varying molarities of carrier iodide in the form of potassium iodide in total volume of 1 ml of Tris Cl buffer at various pH levels. After 3 ml of the buffer was placed in the cathode compartment the constant voltage was turned on. The current was held at 50 μA until the voltage required to maintain this value reached .8V; this generally took twelve to twenty-five minutes. The voltage was then held at .8V and the current allowed to drop off. At the end of 180 minutes the current was usually 15-20 μA.

When reactions were conducted at room temperature an overall decrease in yield of 7.5% was noticed. It was also noted that at room temperature evaporation of the reaction mixture was a problem. Due to possible radiation contamination hazard when radioiodine was volatilized

all further reactions were carried out at 0-5° in a well ventilated fume hood.

Various molar ratios of carrier iodide/bleomycin were introduced but the yield of radioiodinated bleomycin was observed to be less than expected. At a ratio of KI:bleomycin of 8:1, a yield of only 47.2% was obtained after 180 minutes of electrolysis. This confirms the results of Covelli and Wolff who concluded that the incorporation of iodine into histidyl residues occurred at a fixed rate regardless of the carrier iodide molar increase,[94] as long as a sufficient amount was present to allow a 1:1 iodide to histidine relation. It was also pointed out that excess iodine may in fact decrease iodination of a polypeptide by an oxidative action on the molecule.[94]

With these observations in mind the carrier iodide:bleomycin molar ratio was maintained at 1:1 during the subsequent reactions to obtain optimum radioiodination.

A series of experiments were conducted to establish the critical pH at which bleomycin could be radioiodinated with optimum yield. The interdependence of pH and time were observed and are presented on table 3 and figure 9.

The results indicated that maximum yields were obtained at a very narrow pH range with a peak at 7.4 if electrolysis is conducted for 180 minutes.

A simple experiment to resolve the effects of voltage on the process was carried out at four different voltage levels. The formation of iodate (IO_3^-) is always a problem in both chemical and electrolytic iodination procedures. As pointed out earlier, this

Table 3

Effects of pH and Time on Elctrolytic Iodination of Bleomycin Complex

pH	Yield (a)
7	50.0 ± 4.6% (b)
7.2	54.2 ± 4.0%
7.3	72.1 ± 5.3%
7.4	91.3 ± 0.8%
7.5	83.9 ± 7.6%
7.75	73.9 ± 6.9%
8.0	64.5 ± 5.4%
8.5	54.0 ± 2.9%
8.75	46.1 ± 8.0%

(a) 180 min reaction time

(b) mean ± S.D. of 3 runs

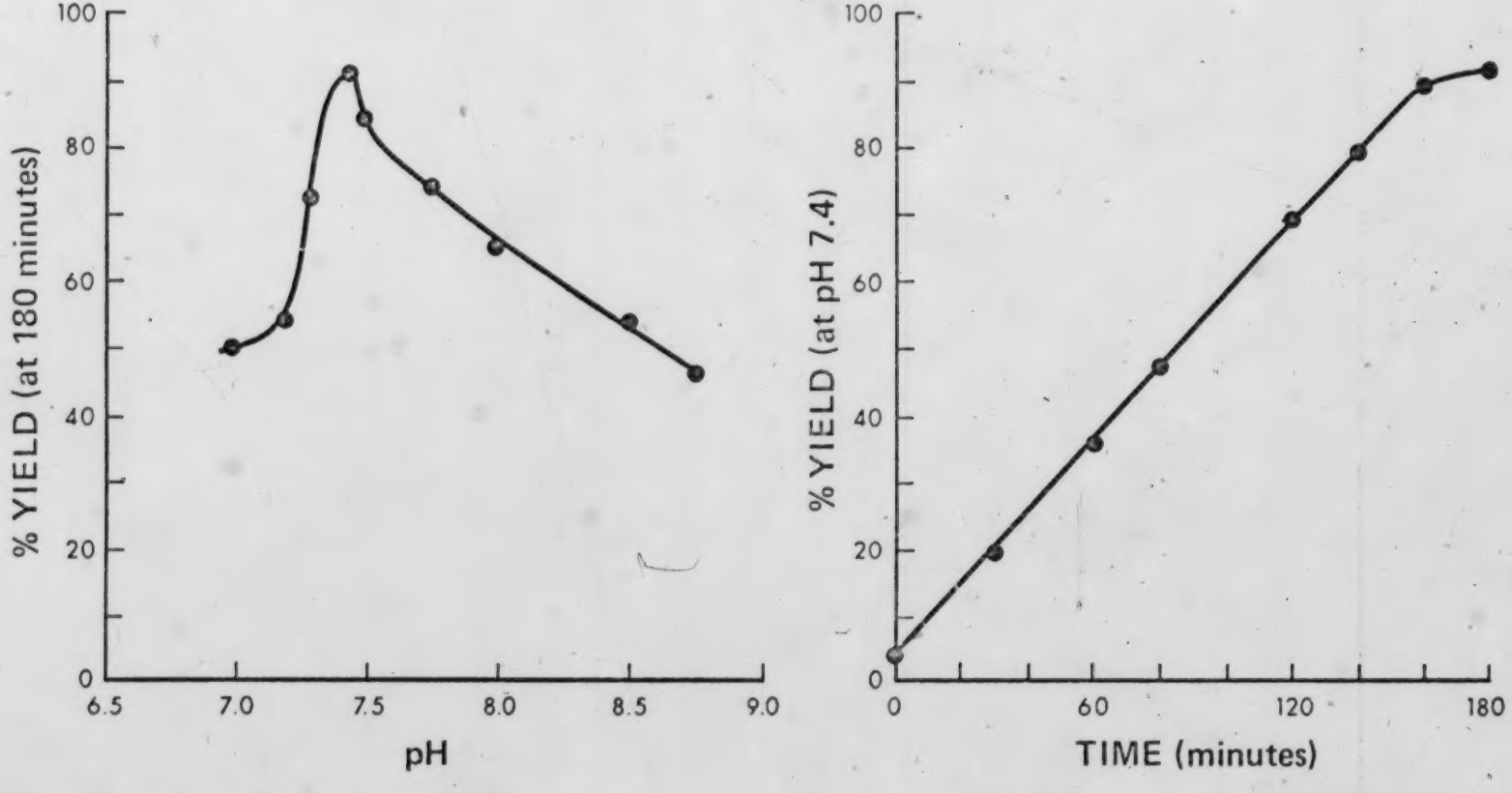
Time (Min)	Yield (a)
0	4.2 ± 1.3% (b)
30	19.1 ± 2.1%
60	35.1 ± 2.1%
75	45.8 ± 3.4%
120	68.0 ± 3.0%
140	78.1 ± 2.6%
160	88.2 ± 1.6%
180	91.3 ± 0.8%

(a) at pH 7.4

(b) mean ± S.D. of 3 runs

Figure 9

Effects of pH and Time on Electrolytic Labeling of Bleomycin Complex



product irreversibly binds the iodine and removes it from further recycling and thus reoxidation and can be represented as follows.

$$I_2 + OH^- \quad HOI + I^-$$

$$HOI \quad H^+ + IO^-$$

$$3OI^- \quad IO_3^-$$

Using the small Sephadex G-10 columns and utilizing the apparatus that was set up for the qualitative assays of the reaction mixtures, it was possible to distinguish between the iodobleomycin, iodate and iodide. The radioactive bleomycin was eluted first at the void volume and the smaller iodate molecule next at 1.5 times the void volume followed by the iodide at twice to 2.5 times the elution volume. To obtain this resolution it was necessary to adjust the flow rates to less than .1 ml per minute. In this manner the three peaks were collected separately and aliquots could then be analyzed for ^{125}I radioactivity.

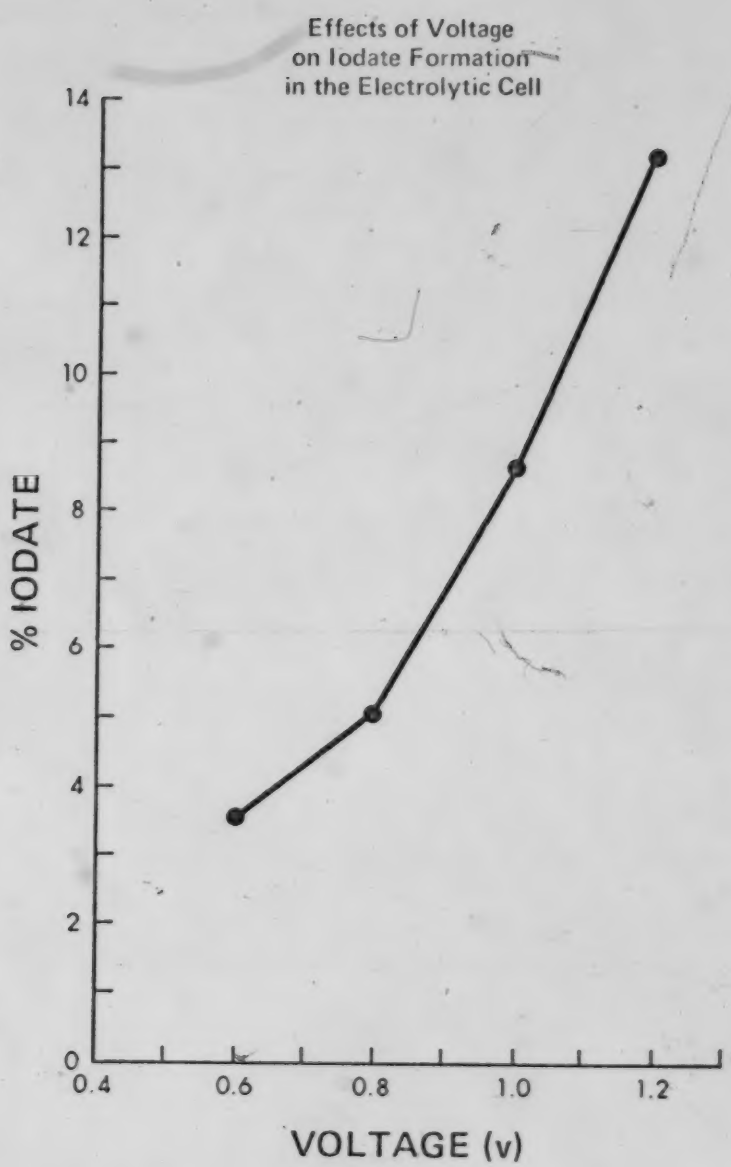
After 120 minutes of electrolysis under the optimum conditions but at voltages of .6, .8, 1.0 and a maximum of 1.2 volts, aliquots of the reaction mixture were removed from the cell and applied to the Sephadex G-10 columns. After elution and analysis of these peaks it was found that the levels of iodate formation increased dramatically with increases in voltage; as indicated in figure 10.

Effects of Voltage on Iodate Formation in Electrolytic Cell (a)

Voltage	% Iodate
.6 V	3.6 ± .03
.8 V	5.1 ± .4
1.0 V	8.7 ± 2.3
1.2 V	13.2 ± 2.1

(a) Mean ± S.D. of two runs

Figure 10


Effects of Voltage
on Iodate Formation
in the Electrolytic Cell
% IODATE
14
12
10
8
6
4
2
0
0.4
0.6
0.8
1.0
1.2
VOLTAGE (v)

It appeared from these results that the .8V maximum chosen arbitrarily and utilized by Sammon[88] was, in fact, the limit to which the voltage could be elevated to, and still maintain low iodate concentrations.

B. Radiochemical Purity of ^{125}I Iodobleomycin Complex and A_2 Sub-fractions

The preceding pages described the optimum parameters for electrolytic iodination of bleomycin. These parameters were established by chromatographic analysis of the reaction mixture at various time intervals. A blank reaction was conducted under optimum conditions, with the deletion of the radioiodine, to ascertain if molecular degradation of the bleomycin molecule was occurring during electroysis. After 180 minutes reaction time the chromatographic results, on tlc and developed in 10% ammonium acetate:methanol 1:1, revealed identical migrational patterns to unreacted bleomycin complex and A_2.

A blank reaction was also conducted complete in every component except for the deletion of bleomycin, to determine if a radiolabel was being acquired by any of the reaction mixture components. On chromatographic analysis in excess of 99% of the activity migrated as free radioiodine. From repeated experiments it was determined that the radioiodination of bleomycin A_2 sub-fraction and complex was optimum under the following conditions:

pH - 7.4 Tris Cl buffer with .1M NaCl

Temperature - 0°C to 5°C

Time - 180 minutes

Carrier iodide:bleomycin ratio - 1:1

$Na^{125}I$ - 50 μCi to 2 mCi

Current - 50 μA

Voltage - 800 mV

A small portion of the reaction mixture was also removed from the cell after ten minutes of mixing the components without the voltage being turned on. Chromatographic analysis of this gave a "zero electrolysis time" labeling value and an indication if any spontaneous labeling was occurring, as shown in table 4 and mean value of 4.2% resulted.

The Eastman chromagrams and Gelman TLC were used for the quantitative labeling results at the various time intervals while the silica-coated glass plate system was run on the final product after 180 minutes of electrolysis. Using ethanol as the solvent the results of the Eastman and Gelman methods were practically identical and served as correlative techniques. In these two procedures the iodo-bleomycin had an R_f of zero or stayed at the origin while the unbound ^{125}I migrated with the solvent front and generally localized at an R_f of .85 to .9 as seen in figure 11.

The characteristics of the reaction product were studied by spotting small aliquots of the reaction mixture on the glass support silica gel thin layer chromatographic system after 180 minutes of electrolysis. A comparison of the migrational behaviour of this material to the unlabeled bleomycin in the 10% ammonium acetate: methanol (1:1) solvent system was carried out. After developing to 10 cm and drying the various spots could be visualized using the short wave uv light. One centimeter segments were then scraped off the plates and analyzed for radioactivity in a gamma spectrometer. The

Table 4

Characteristics of Radioiodinated Bleomycin Complex on Silica Gel tlc

Segment No.	CPM(b)	% (c)	CPM(b)	% (c)	CPM(b)	% (c)
1	160		170		23	
2	219		268		116	
3	347		489		117	
4	96917	67.1%	63643	63.0%	75527	58.8%
5	2515	1.7%	1515	1.51%	2590	2.1%
6	214		174		196	
7	45005	31.2%	35863	35.49%	48228	39.1%
8	414		201		312	
9	14675		8379		11976	
10	20		67		57	
Total Radio-activity on plate	160486	89.99%	110769	91.2%	136142	90.6%
Total radio-activity of bleomycin complex	144437		101021		123345	

(a) developed in 10 cm in 10% ammonium acetate:methanol (1:1) at 20°C

(b) background corrected

(c) expressed as a percentage of total radioiodine bound to the bleomycin complex.

Figure 11

Characteristics of ^{125}I Iodobleomycin Complex on TLC Systems

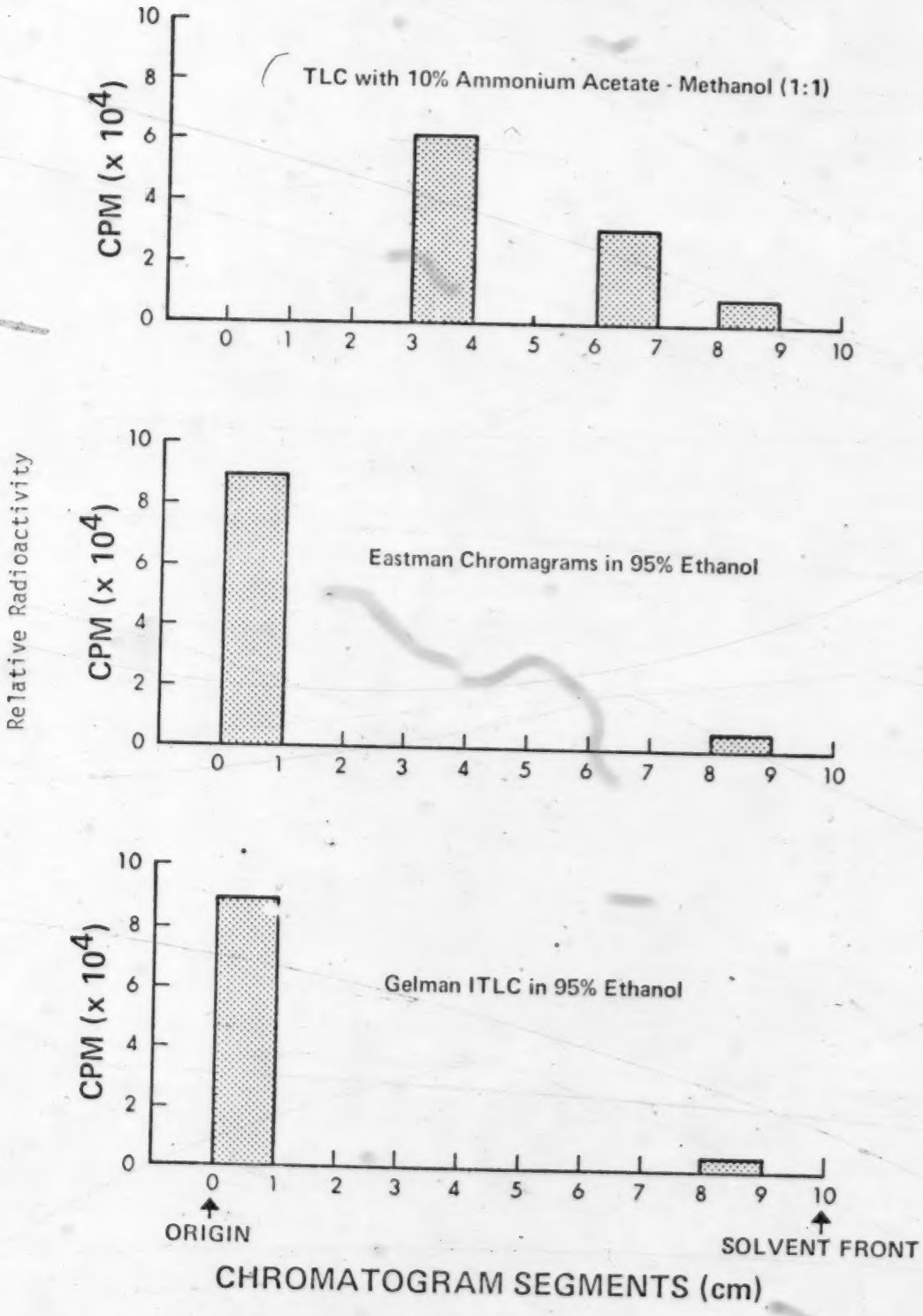
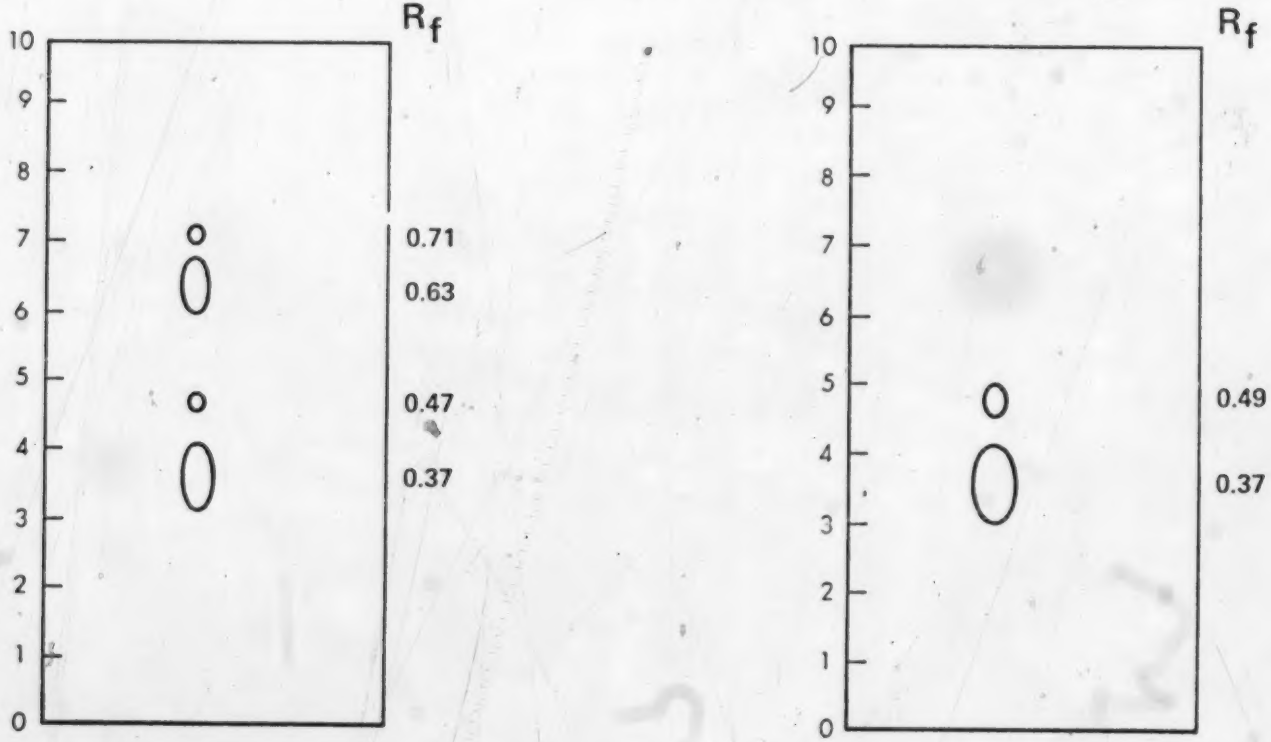

TLC with 10% Ammonium Acetate - Methanol (1:1)
Eastman Chromagrams in 95% Ethanol
Gelman ITLC in 95% Ethanol
CPM (x 10^4)
Relative Radioactivity
0 1 2 3 4 5 6 7 8 9 10
ORIGIN
SOLVENT FRONT
CHROMATOGRAM SEGMENTS (cm)

Figure 12



Characteristics of ^{125}I Iodobleomycin Complex and ^{125}I Iodobleomycin A_2 on TLC (a)(b)

(a) Developed with 10% ammonium acetate:methanol (1:1) at 20°C.
(b) Visualized with short wave uv lamp.

iodobleomycin acted in an identical manner as the unlabeled material chromatographically as illustrated in figure 12.

When the ^{125}I-iodobleomycin complex was used, spots at Rf of .37, .47, .63 and .71 were visualized. The results using radioiodinated bleomycin A_2 yielded one large spot at Rf of .35 with a small diffuse spot at .49. There were no degradation products exhibited within the sensitivity of this analytical method. These results indicated that no gross chemical changes were undergone by the bleomycin molecules as a result of the radioiodination conditions.

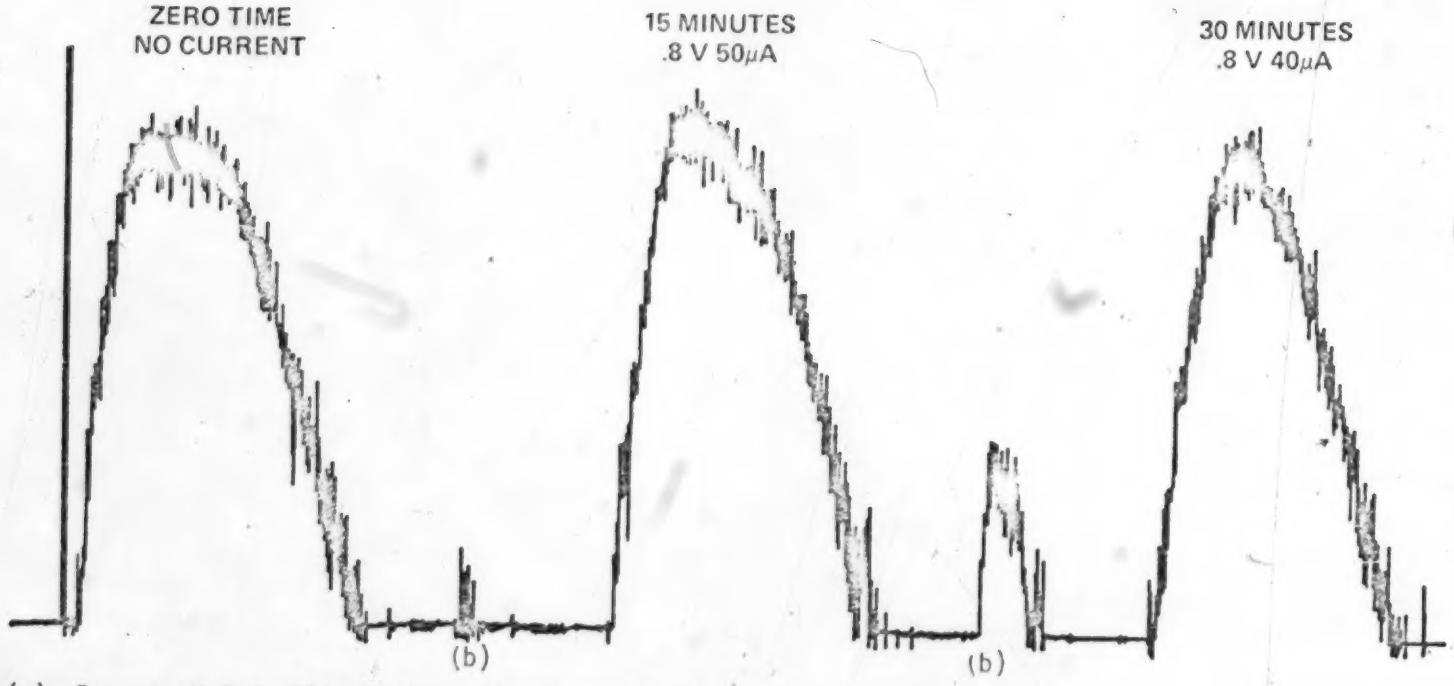
On assay for radioactivity the following data on table 4 indicates the fractions which were labeled. In the iodobleomycin complex, 62.9 ± 4.2% of the total radioiodine bound to the complex was associated with the bleomycin A_2 fraction (segment number 4) and that 90% of the ^{125}I activity was bound to the complex.

C. Qualitative Elution Profile of Radioiodinated Bleomycin A_2 of Sephadex G-10 Column

Figure 13 and 14 show a representative elution profile obtained serially during the radiolabeling process of the bleomycin A_2. The progress of the labeling could be followed by applying 25 µl to 50 µl aliquots of the cell reaction mixture to small Sephadex G-10 columns and monitoring the eluate radioactivity with a NaI (TI) crystal and appropriate electronics. The iodobleomycin eluted off the column at the void volume; the iodate being a smaller molecule was retarded in the gel pores and appeared at 1.5 times the void volume. The free iodide appeared at twice the elution volume.

Figure 13

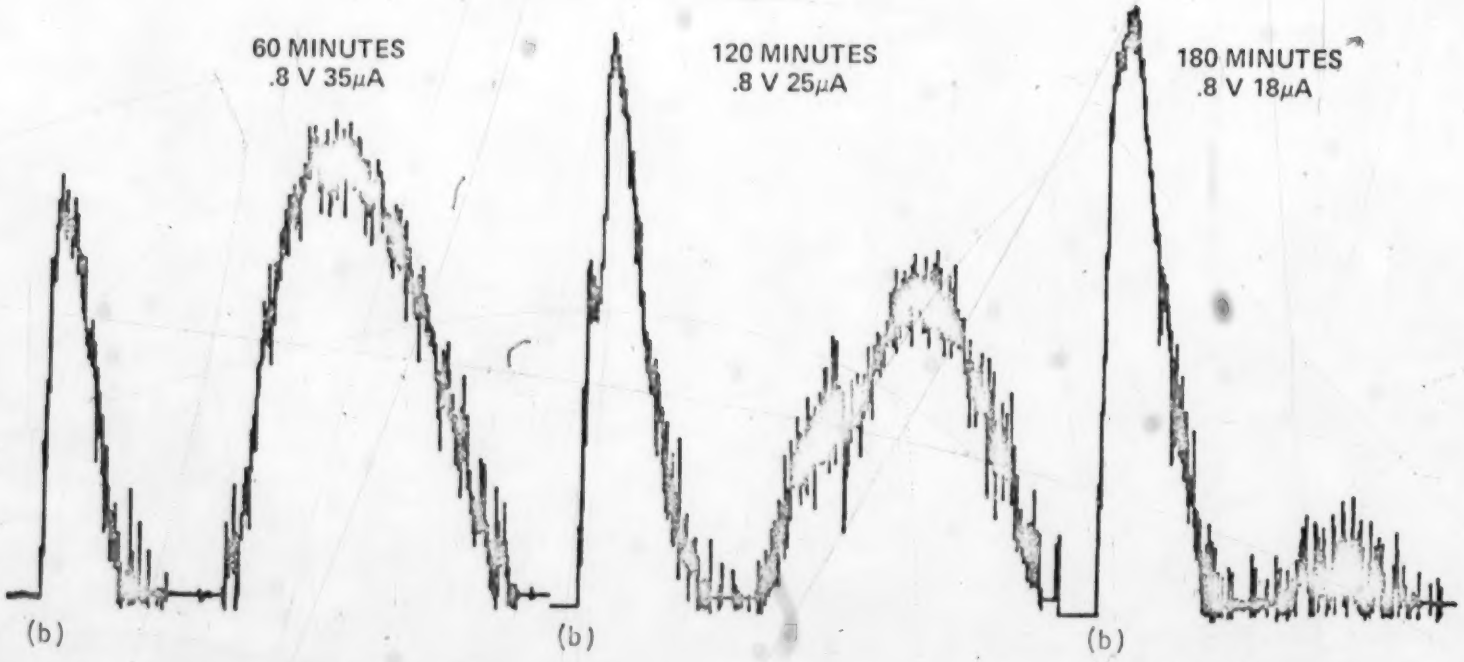
SEPHADEX G-10 ELUTION PROFILE
FOLLOWING RADIOIODINATION OF
BLEOMYCIN A_2 (a)



(a) Run at pH 7.4, 0°C, maximum of .8V and 50 μA.
(b) Elution volume of column 3.4 ml.

Figure 14

SEPHADEX G-10 ELUTION PROFILE
FOLLOWING PROGRESS OF RADIOIODINATION
BLEOMYCIN A_2 (a)



(a) Run at pH 7.4, 0°C, maximum of .8V and 50 μA.
(b) Elution volume of column 3.4 ml.

The "zero electrolysis time" exhibits a broad large peak that is indicative of unbound iodide and was observed at an elution volume of 10.2 ml. As the time intervals progress, the peak appearing at the void volume of 3.4 ml becomes more prominent until at 180 minutes the free iodide radioactivity has diminished to very low levels and the majority of the radioactivity is associated with the bleomycin.

D. Purification of the Radioiodinated Bleomycin A_2

The labeled product of the electrolytic reaction could be successfully purified by passage through Sephadex G-10 to remove the iodide and iodate impurities. This method was undesirable due to the large dilution and the use of the Dowex 1 x 4 anion exchange resin proved to be a more efficient method. After passage through the 2 mm bead bed, and adequately back washing, the final product was assayed on Eastman and Gelman ITLC. On both systems an average of 4.23% of the activity was associated with the solvent front as free iodide, as shown in table 5. About 95.7% of the radioactivity was associated with the bleomycin A_2. Unfortunately there was an overall loss of 28% of the product on the syringe walls, fritted glass fittings and adsorbed to the resin beads. This was determined by assaying the radioactivity of an aliquot of the reaction product before and after passage through the anion exchange resin. Further purification in preparation for the biological studies involved passing the ^{125}I-iodobleomycin solution through a .22 micron millipore filter. Unfortunately millipore filtration led to a further loss of up to

Table 5 .

Results of Dowex 1 x 4 Anion Exchange
Extraction of Unbound $Na^{125}I$ from ^{125}I-Bleomycin A_2 Reaction Mixture

Pre Dowex 1x4 Purification

Reaction	Eastman Chromagrams (a) Total Plate CPM	CPM at R_f .85	% Unbound $Na^{125}I$	Gelman ITLC (a) Total Plate CPM	CPM at R_f .85	% Unbound $Na^{125}I$
1	176,417	19,582	11.1	208,411	22,717	10.9
2	211,419	19,661	9.3	226,101	19,671	8.7
3	168,787	17,554	10.4	201,787	18,766	9.3

Post Dowex 1x4 Purification

1	167,806	6,544	3.9	192,427	7,120	3.7
2	142,912	6,860	4.8	178,777	7,330	4.1
3	183,413	7,703	4.2	191,909	9,019	4.7

(a) Solvent is 95% ethanol

47.6% of the product. Since the bleomycin molecule is thermally stable,[6] the iodobleomycin could be terminally autoclaved to bypass this costly filtering step if used for human clinical studies.

E. Stability of the Iodinated Bleomycin A_2 and Complex

Both the labeled bleomycin A_2 and labeled complex were stored at pH 7.4, at temperature of 4°C and 20°C for thirty days. At daily intervals .015 ml aliquots of each solution was spotted on Eastman chromagrams and developed to 10 cm in 95% ethanol. The results on table 6 indicate a slightly greater dehalogenation at room temperature than when the material was refrigerated. The refrigerated material lost about .7% of the label per day while the material at room temperature lost about 1% per day. There appeared to be no difference in the loss of the radioiodine between the bleomycin A_2 sub-fraction and the entire bleomycin complex. At the end of the thirty day period, .025 ml aliquots were removed from each vial and spotted on the conventional glass support tlc plates. These were developed in the standard method as described and visualized with a short-wave uv lamp. Spots were detected at R_f of .35, .5 and a large spot covering .68 to .72, on the plate that was used for assaying the bleomycin complex, and at R_f values of .35 and .5 with the bleomycin A_2. These spots were identical to the chromatographic behaviour of bleomycin A_2 and complex prior to radioiodination. Using this system of chromatographic analysis there was no indication of degradation or molecular fragmentation due to hydrolysis.

Table 6

Stability of Electrolytically labeled Iodobleomycin Complex and A_2 at 4°C and 20°C (a)(b)(c)

	Bleomycin Complex		Bleomycin A_2	
Day	20°C	4°C	20°C	4°C
1	100°	100°	100°	100°
2	98.1 ± .4	97.4 ± 2.1	99.2 ± 1.2	98.6 ± 1.1
3	96.7 ± 1.1	96.5 ± 1.3	97.4 ± .4	96.8 ± 1.1
4	94.3 ± .9	94.6 ± .9	95.4 ± 1.8	96.2 ± .9
5	93.8 ± 1.6	94.2 ± .7	93.8 ± 1.4	95.1 ± 1.6
6	92.7 ± .6	93.9 ± 1.1	92.6 ± 2.1	93.4 ± .6
7	92.5 ± 1.6	92.8 ± 2.5	92.4 ± .9	92.6 ± 1.2
15	85.4 ± .7	89.9 ± 1.6	86.3 ± .7	87.4 ± 1.2
21	79.6 ± 2.8	84.4 ± .6	81.3 ± .9	83.6 ± 2.1
30	68.4 ± 1.6	80.1 ± 1.4	72.8 ± 1.4	79.8 ± .9
Total loss	31.6%	19.9%	27.2%	21.2%
Loss/ day	1.05%	.66%	.90%	.70%

(a) Assays were on Eastman Chromagrams using 95% ethanol

(b) All values corrected for physical decay and background

(c) All values are expressed as a percent of original label which is considered 100% and are the mean ± S.D. of triplicate runs

The thermal stability of three different reaction products of both labeled complex and A_2 were tested after the free iodide had been removed with the Dowex 1 x 4 anion exchange resin. The ^{125}I-iodobleomycin in closed, vented vials was placed in a boiling water bath at 100°C for sixty minutes. After incubation for sixty minutes at 100°C the vials were cooled and the solution was assayed on Eastman Chromagrams in 95% ethanol for radiochemical purity and also on glass support tlc for the detection of degradation products. The results are shown in the following table.

Thermal Stability of Electrolytically Radioiodinated Bleomycin Complex and A_2 at 100°C for 60 Minutes (a)

Bleomycin Complex	Percent of initial Bleomycin-bound iodine After 60 min at 100°C
1	86.2
2	89.7
3	87.4
Bleomycin A_2	
1	89.6
2	88.4
3	90.1

(a) All solutions were at pH 7.4

As shown in the above table the ^{125}I-bleomycin complex dehalogenated on the average of 12.2 ± 1.9% and the bleomycin A_2 lost 10.6 ± 1% of the incorporated radioiodine after heating. The migrational behaviour on the tlc plates was identical with the characteristic

unlabeled bleomycin complex and bleomycin A_2, indicating that no gross degradation had occurred.

F. Stability of Iodobleomycin A_2 in the Presence of Cu^{++} Ions

It is well documented that bleomycin exists as a natural copper chelate[3] and preferentially chelates copper to a greater extent than other cations.[5] An experiment was conducted to challenge the carbon-iodine bond and to determine if copper ions could displace the radioiodine label on the bleomycin molecule. This would have future implications in in vivo studies, as the iodine may, in fact, be removed during biological metabolism by the various cations present in living organisms.

This was accomplished by adding an equimolar solution of Cu^{++} in the form of copper sulfate to a purified radioiodinated bleomycin A_2 solution and mixing vigorously with a magnetic stirring bar for thirty minutes. A .015 ml portion of the resultant solution was then chromatographed on glass support tlc plates and assayed on Eastman chromagrams for radiochemical purity. After development and drying of the glass support tlc the spot at R_f .35 was visualized, using a uv lamp. There was no additional spots visible. After scraping, 1 cm segments off the plate and assaying for radioactivity it was noted that the majority of the activity was associated with this spot and indicated the presence of ^{125}I-bleomycin A_2. The segments between R_f .4-.75 contained a level of radioactivity greater than normally seen with residual iodide adsorbing to the silica. The usual activity at an R_f of .85, indicating free iodide, was absent.

The Eastman chromagrams were developed in the usual manner in 95% ethanol and 1 cm segments were assayed for radioactivity individually. Again the majority of the activity was associated with the ^{125}I-bleomycin A_2 at the origin. An additional peak of radioactivity was also noted at R_f .35 but the free iodide band at R_f .85 was absent. Although the ^{125}I-iodobleomycin remained intact during this experiment both chromatographic systems, however, indicated the presence of a small amount of an unknown species. This unknown molecule appeared to be less polar than iodobleomycin and bound the free Na^{125}I in the solution. This complex formation was not investigated any further. The experiment had shown that the ^{125}I iodobleomycin was quite stable in the presence of Cu^{++} ions, suggesting that the iodine was covalently bound rather than weakly chelated.

G. Technetium 99m Labeling of Bleomycin

Bleomycin complex was labeled with technetium using a prepared stannous pyrophosphate kit as the source of a reducing agent.[47] To supply approximately 4.2 µg of tin, .076 ml of the stock pyrophosphate solution was used, and mixed at 22°C for five minutes with 4.1 mg of bleomycin complex dissolved in .5 ml of pertechnetate. The resultant solution was chromatographed on the three systems, assayed and the results are presented in table 7.

The glass support tlc, after development in 10% ammonium acetate: methanol (1:1), was placed under a uv lamp and spots at R_f of .35, .5, and .68 were visualized. These R_f values corresponded to the migrational behaviour of bleomycin complex in this solvent system.

Ten 1 cm segments were scraped from the plate and on subsequent assay 80.4% of the radioactivity was associated with the three spots.

The bleomycin labeling technique, using ^{99m}Tc-pyrophosphate, was conducted on a number of occasions with varying success. The unpredictable results, which ranged from 42% to 86% ^{99m}Tc bound bleomycin, yielded some products that were unacceptable for biological studies. Only the highest yield ^{99m}Tc bleomycin products were used; the remainder were discarded.

Due to the short half life of ^{99m}Tc, stability studies were not conducted on this material. Prior to injection into animal models the reaction mixture was passed through Dowex 1 x 4 anion exchange resin to eliminate the majority of the free pertechnetate as shown in table 8. This yielded a final ^{99m}Tc-bleomycin complex solution of approximately 96%.

Table 7

Radiochemical Purity of ^{99m}Tc Bleomycin Complex

	Gelman ITLC (a)		Eastman Chromagrams (a)	
	% at Rf 0	% at Rf .9	% at Rf 0	% at Rf .9
1	80.81	18.2	84.7	14.3
2	81.88	16.9	82.1	17.2
3	83.22	15.9	83.4	15.9
Average label	82%		83.4%	

(a) Developed in 95% ethanol at 22°C.

Table 8

Dowex 1 x 4 Anion Exchange

Extraction of Unbound ^{99m}Tc from ^{99m}Tc-Bleomycin Complex (a)

Pre Dowex 1 x 4 Purification

Reaction	Total Plate CPM	CPM at Rf .9	% Unbound ^{99m}Tc
1	476,076	82,837	17.4
2	394,192	73,320	18.6
3	370,159	62,357	16.9

Post Dowex 1 x 4 Purification

1	292,472	12,283	4.2
2	314,416	11,319	3.6
3	216,894	9,326	4.3

Average 96% yield

(a) Developed on Gelman ITLC with 95% ethanol at room temperature

VI. Animal Studies

A. Preliminary Tissue Distribution

A preliminary uptake and distribution study, using ^{125}I-bleomycin A_2, was undertaken to ascertain the parameters for a more detailed experiment. At time intervals of one and one half, three and six hours after injection, via the tail vein, of approximately 15 µCi of the radioiodinated bleomycin, tumor-bearing mice were sacrificed, and samples of a variety of organs were obtained and assayed. The assays of all tissue samples were determined using the Searle 1195 gamma spectrometer. Tissue samples of lung, liver, spleen, kidney, leg muscle, testes, brain, intestine, femor, heart, skin, tumor and blood were obtained, washed in normal saline, blotted dry, and assayed in individual glass counting vials. Table 9 exhibits the results and indicates that low levels of radioactivity were detected in the bone, heart and gut. Activity in the bone, skin and heart may be associated with residual blood activity. The results of these assays were expressed as counts per minute per milligram (cpm/mg) to obtain relative ratios between tissues only. The lung, liver, spleen, testes, muscle, tumor and blood were chosen as the organs of interest for further study.

The carcasses were disected into head, body and tail and these portions were assayed individually for radioactivity in the small animal whole body counter. No abnormal elevation of radioactivity was noted in the head, which would indicate free iodine being taken up by the thyroid if the nuclide was being released during *in vivo*

Table 9

Preliminary Tissue Distribution of ^{125}I-bleomycin A_2 in Tumor Bearing Mice (a) (b)

Tissue	1.5 Hours	3 Hours	6 Hours
Liver	58 ± 6	21 ± 3	10 ± 3
Spleen	31 ± 4	12 ± 3	6 ± 1
Lung	96 ± 10	43 ± 4	9 ± 2
Kidney	547 ± 81	212 ± 31	161 ± 14
Testes	146 ± 18	127 ± 16	61 ± 11
Muscle	97 ± 11	44 ± 5	10 ± 2
Tumor	241 ± 25	196 ± 18	66 ± 9
Blood (c)	36412	4632	714
Brain	26 ± 2	12 ± 2	4 ± 1
Intestine	43 ± 3	18 ± 3	10 ± 2
Bone	74 ± 6	27 ± 2	14 ± 4
Heart	36 ± 4	21 ± 2	9 ± 1
Skin	112 ± 10	101 ± 11	64 ± 5

(a) Expressed as cpm/mg of mean of 3 animals ± S.D.

(b) Fifteen microcuries in 0.1 ml was injected into each animal intravenously.

(c) cpm per 0.1 ml whole blood.

metabolism. A lack of significant radioactivity in the tail indicated that there was little residual ^{125}I-iodobleomycin retained at the site of injection.

B. Tissue Distribution of ^{125}I-bleomycin Complex in Normal Mice

This study was conducted on 20-25 gm young male IRC Swiss mice at the designated time intervals after injection of ^{125}I-bleomycin complex. The injected material was prepared by the electrolytic iodination procedure and was deemed 97% pure by chromatographic assay. The solution contained 25 µCi per 0.1 ml of a specific activity of 70 µCi/mg. Groups of six mice were sacrificed at one, two, three, four, six, twelve and twenty-four hours post-injection by ether asphyxiation. A volume of 0.1 ml of whole blood was immediately removed by direct cardiac puncture and the designated organs were excised, rinsed, blotted dry, weighed and placed in glass counting vials. These samples were then assayed for radioactivity in the appropriate gamma spectrometer. The results are illustrated in table 10 and figure 15.

One hours post-administration of the ^{125}I-bleomycin complex the blood, kidneys, lung and testes showed high levels of radioactivity. This activity rapidly decreased as the material cleared from all tissues with the exception of the kidney which retained .5% of the injected dose per gram after four hours. Elevated levels of radioactivity were noted in this organ throughout the study period. The assayed radioactivity in the other tissue samples reached low levels

Table 10

Tissue Distribution and Uptake of ^{125}I-Bleomycin Complex in Normal Male IRC Mice (a)(b)

Tissue	Time Post-Intravenous Administration (hours)				
	1	2	3	4	6
Liver	0.19 ± 0.04	0.11 ± 0.03	0.06 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
Spleen	0.10 ± 0.05	0.03 ± 0.0	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.00
Lung	0.45 ± 0.04	0.22 ± 0.04	0.21 ± 0.03	0.05 ± 0.01	0.02 ± 0.00
Testes	0.47 ± 0.17	0.41 ± 0.06	0.34 ± 0.04	0.11 ± 0.01	0.02 ± 0.01
Kidney	2.10 ± 0.41	0.98 ± 0.18	0.57 ± 0.07	0.50 ± 0.05	0.41 ± 0.04
Muscle	0.23 ± 0.04	0.11 ± 0.01	0.06 ± 0.01	0.03 ± 0.01	0.01 ± 0.01
Blood (c)	1.29 ± 0.11	0.83 ± 0.06	0.13 ± 0.02	0.06 ± 0.01	0.02 ± 0.00

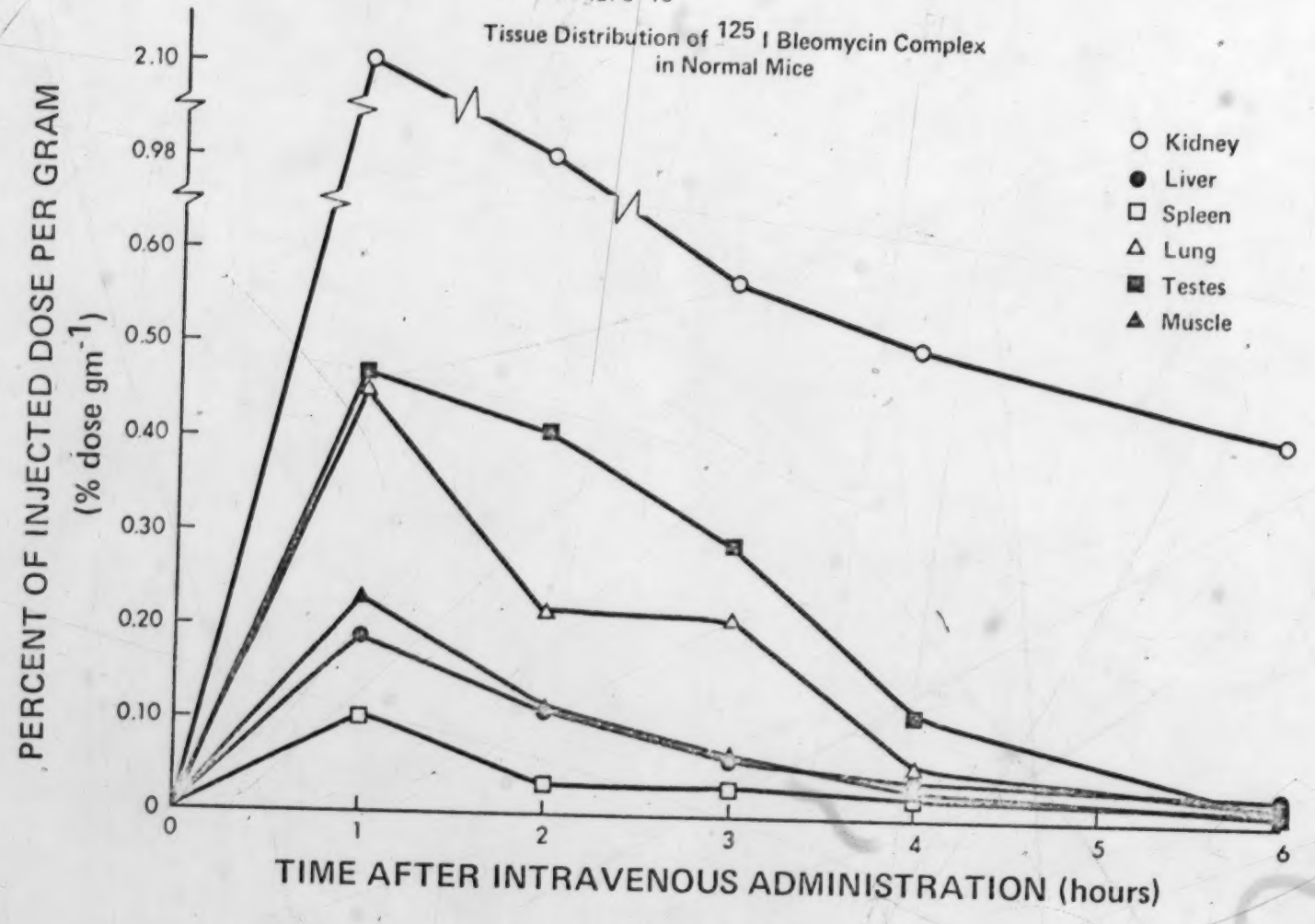
(a) Mean of six animals ± S.D. at each time interval and expressed as percent dose per gram of tissue sample

(b) Each animal injected with 25 µCi of ^{125}I-bleomycin complex

(c) Expressed as percent dose per 0.1 ml of whole fresh blood

Figure 15

Tissue Distribution of ^{125}I Bleomycin Complex in Normal Mice



at the four hour post-injection time interval and at six hours only 0.02% - 0.03% of the injected dose per gram of sample remained.

C. Tissue Distribution of ^{125}I-Bleomycin A_2 in Normal Mice

The tissue distribution of radioiodinated bleomycin A_2 was then studied in a group of normal mice. This A_2 sub-fraction had been previously prepared by the silica-gel column chromatography separation procedure described earlier and was radioiodinated electrolytically under optimum conditions to yield a product of specific activity of 70 µCi/mg. Portions of 0.1 ml of ^{125}I-bleomycin A_2 were injected, containing 25 µCi, via tail vein into forty-two animals. Groups of six animals were serially sacrificed at the specified time intervals and the blood and excised tissue samples were assayed for radioactivity. Table 11 and figure 16 indicate the results.

Again the blood, kidney, lung and testes exhibit the highest levels of uptake, at one hour post-injection of ^{125}I-bleomycin A_2. The tissue clearance of this material was assayed and very low levels of radioactivity were noted at four post-administration with the radioactivity diminishing to 0.02-0.03% of the injected dose per gram in all tissues except the kidney. This organ retained .44% of the injected dose per gram even after six hours. The levels of distribution of this study with the iodinated A_2 sub-fraction, and those using the iodinated complex were found to be very similar in all aspects. There was no actual significant difference in the two series of distribution studies using normal animals, when the data was subjected to an unpaired t-test at 5% level of significance and 10 degrees of freedom.

Table 11

Tissue Distribution and Uptake of ^{125}I-Bleomycin A_2

in Normal Male IRC Mice (a)(b)

Tissue	Time Post-Intravenous Administration (Hours)				
	1	2	3	4	6
Liver	0.20 ± 0.05	.11 ± 0.02	0.06 ± 0.01	0.04 ± 0.0	.03 ± 0.01
Spleen	0.16 ± 0.03	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.0	.02 ± 0.0
Lung	0.48 ± 0.05	0.22 ± 0.04	0.21 ± 0.04	0.06 ± 0.01	0.02 ± 0.0
Testes	0.51 ± 0.02	0.41 ± 0.09	0.35 ± 0.04	0.10 ± 0.0	0.02 ± 0.0
Kidney	2.10 ± 0.32	0.95 ± 0.15	0.59 ± 0.06	0.50 ± 0.09	0.43 ± 0.04
Muscle	0.23 ± 0.04	0.10 ± 0.01	0.06 ± 0.02	0.04 ± 0.02	0.01 ± 0.0
Blood (c)	1.30 ± 0.05	0.83 ± 0.06	0.11 ± 0.02	0.05 ± 0.01	0.01 ± 0.0

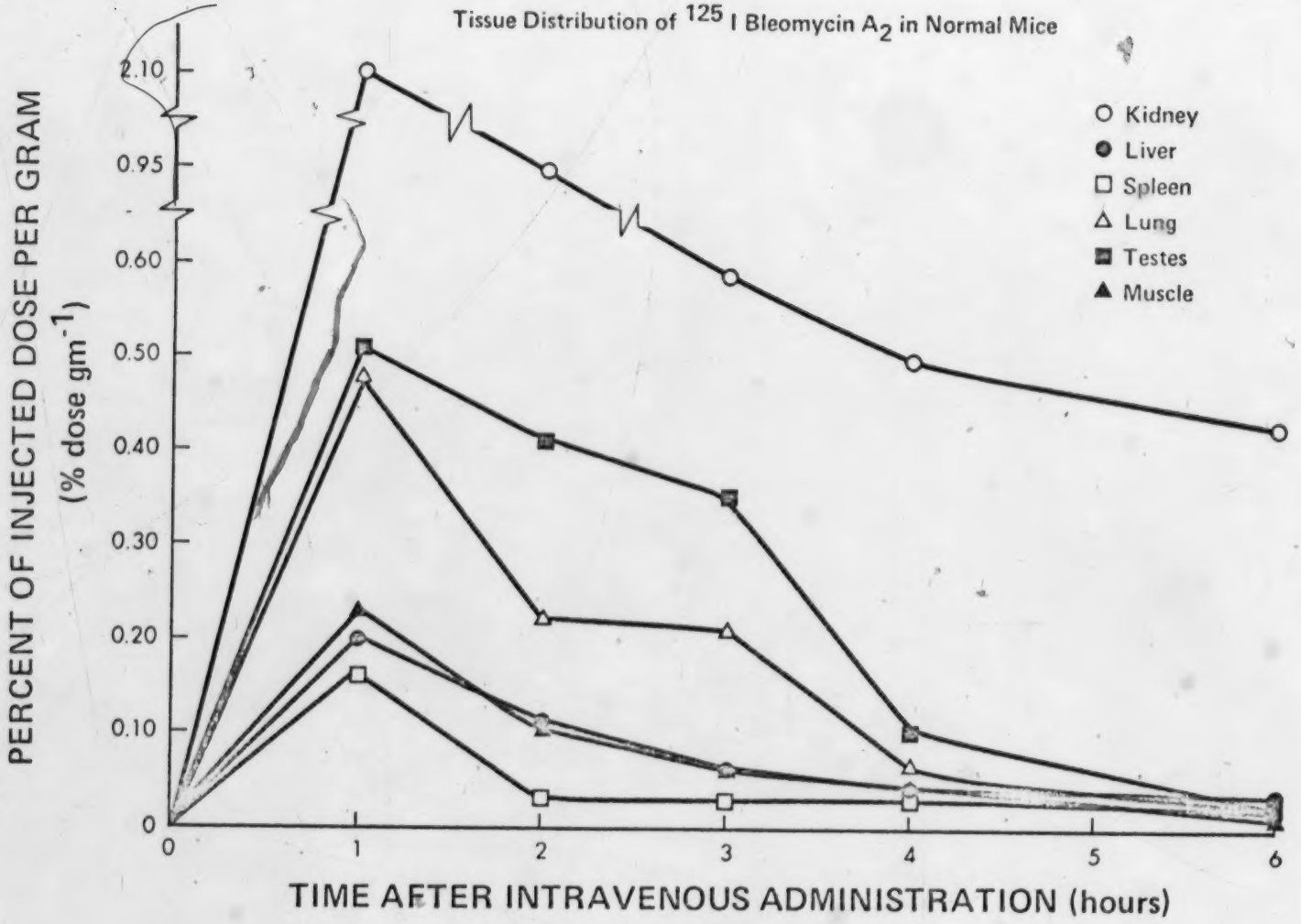
(a) Mean of six animals ± S.D. at each time interval and expressed as percent dose per gram of tissue sample

(b) Each animal was injected with ^{125}I-bleomycin A_2 solution (25 µCi)

(c) Blood was expressed as percent dose per 0.1 ml of whole fresh blood

Figure 16

Tissue Distribution of ^{125}I Bleomycin A_2 in Normal Mice



D. Tissue Distribution of ^{125}I-Bleomycin Complex in Mice Bearing a Solid Form of Ehrlich Ascites Tumor

Male Swiss mice were innoculated with Ehrlich ascites cells in the femoral region and the tumor allowed to incubate in vivo for approximately seven days. A solid non-mobile mass could be palpated at this time. Electrolytically iodinated ^{125}I-bleomycin complex was injected intravenously and at the predesignated time intervals groups of six animals were sacrificed. A 0.1 ml solution of this material contained 12 µCi or a specific activity of 30 µCi/mg. Blood and the various tissue samples were removed and assayed for radioactive uptake.

As noted in table 12 and figures 17 and 18 high and persistent tumor levels were exhibited and at six hours post-injection yielded maximum tumor:blood and tumor:muscle ratios of 8:1 and 16:1 with the exception of the high kidney levels, the other tissues at this time interval had consistently reached the low levels of radioactivity observed in the normal animal distribution studies. The tissue and blood levels decrease rapidly after about two to three hours post-injection.

E. Tissue Distribution of ^{125}I-Bleomycin A_2 in Mice Bearing a Solid Form of Ehrlich Ascites Tumor

A comparative study using the Ehrlich ascites mouse tumor model was conducted using electrolytically iodinated ^{125}I-bleomycin A_2. After intravenous injection of 14 µCi of the labeled compound, groups of six animals were sacrificed at the various time periods.

Table 12

Tissue Distribution and Uptake of ^{125}I-Bleomycin Complex in Male IRC Tumor-Model Mice (a)(b)

Tissue	Time Post Intravenous Administration				
	1	2	3	4	6
Liver	0.24 ± 0.02	0.14 ± 0.01	0.09 ± 0.01	0.05 ± 0.0	0.02 ± 0.0
Spleen	0.13 ± 0.03	0.08 ± 0.01	0.03 ± 0.0	0.02 ± 0.01	0.03 ± 0.0
Lung	0.54 ± 0.1	0.22 ± 0.02	0.18 ± 0.01	0.08 ± 0.0	0.03 ± 0.01
Testes	0.48 ± 0.02	0.40 ± 0.04	0.36 ± 0.03	0.14 ± 0.03	0.05 ± 0.01
Kidney	2.02 ± 0.11	0.99 ± 0.04	0.60 ± 0.06	0.48 ± 0.09	0.46 ± 0.08
Muscle	0.26 ± 0.04	0.11 ± 0.01	0.05 ± 0.00	0.03 ± 0.01	0.01 ± 0.0
Tumor	0.54 ± 0.05	0.53 ± 0.02	0.41 ± 0.03	0.30 ± 0.06	0.16 ± 0.01
Blood (c)	0.22 ± 0.10	0.80 ± 0.06	0.16 ± 0.04	0.05 ± 0.00	0.02 ± 0.0

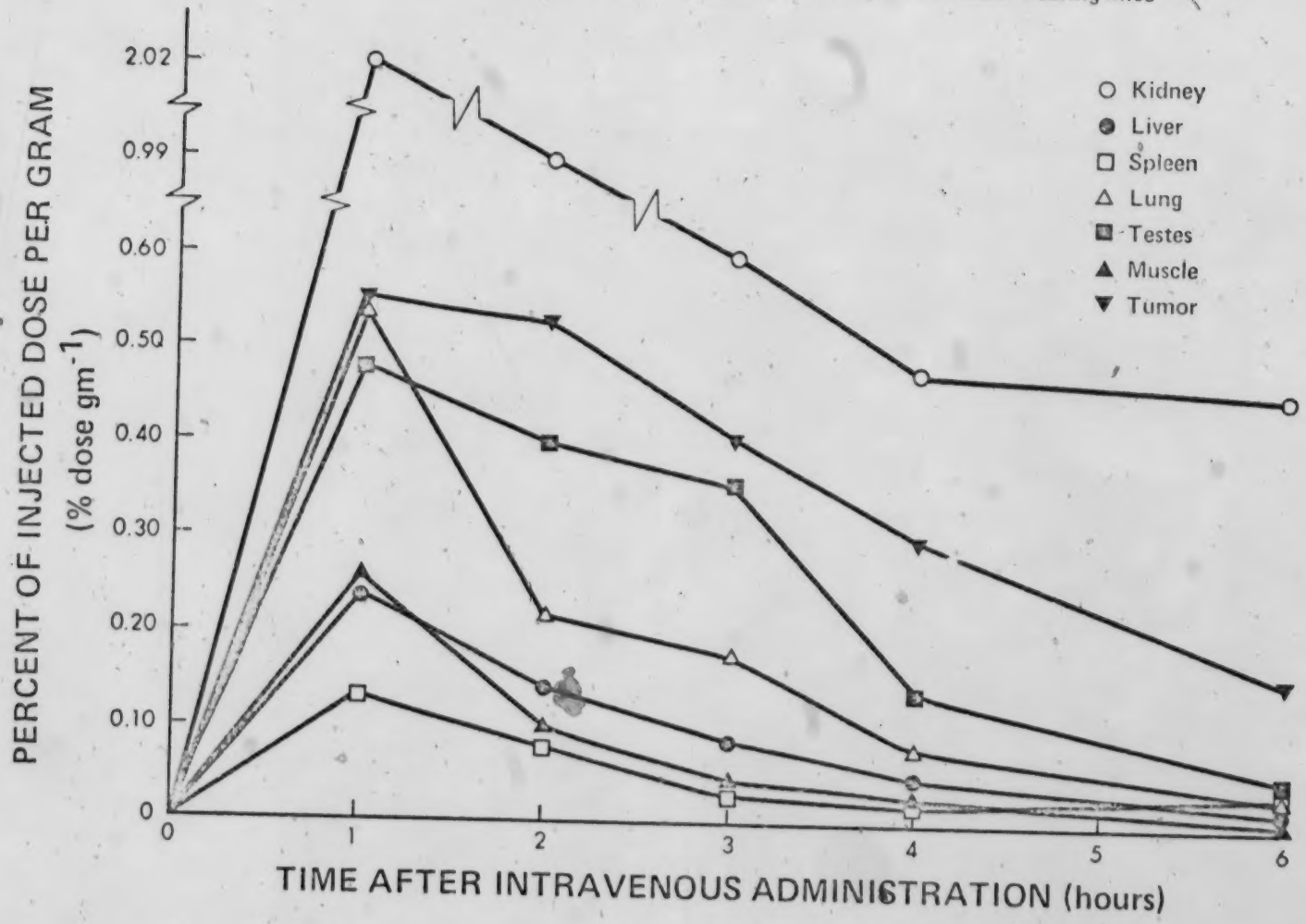
(a) Mean of six animals ± S.D. at each time interval and expressed as percent dose per gram of tissue sample.

(b) Each animal was injected with 12 µCi of ^{125}I-bleomycin complex

(c) Expressed as a percent of total dose per 0.1 ml of whole fresh blood

Figure 17

Tissue Distribution of ^{125}I Bleomycin Complex in Tumor Bearing Mice



Relative Concentrations 0 ^{125}I - Bleomycin Complex in Tumor, Muscle and Blood

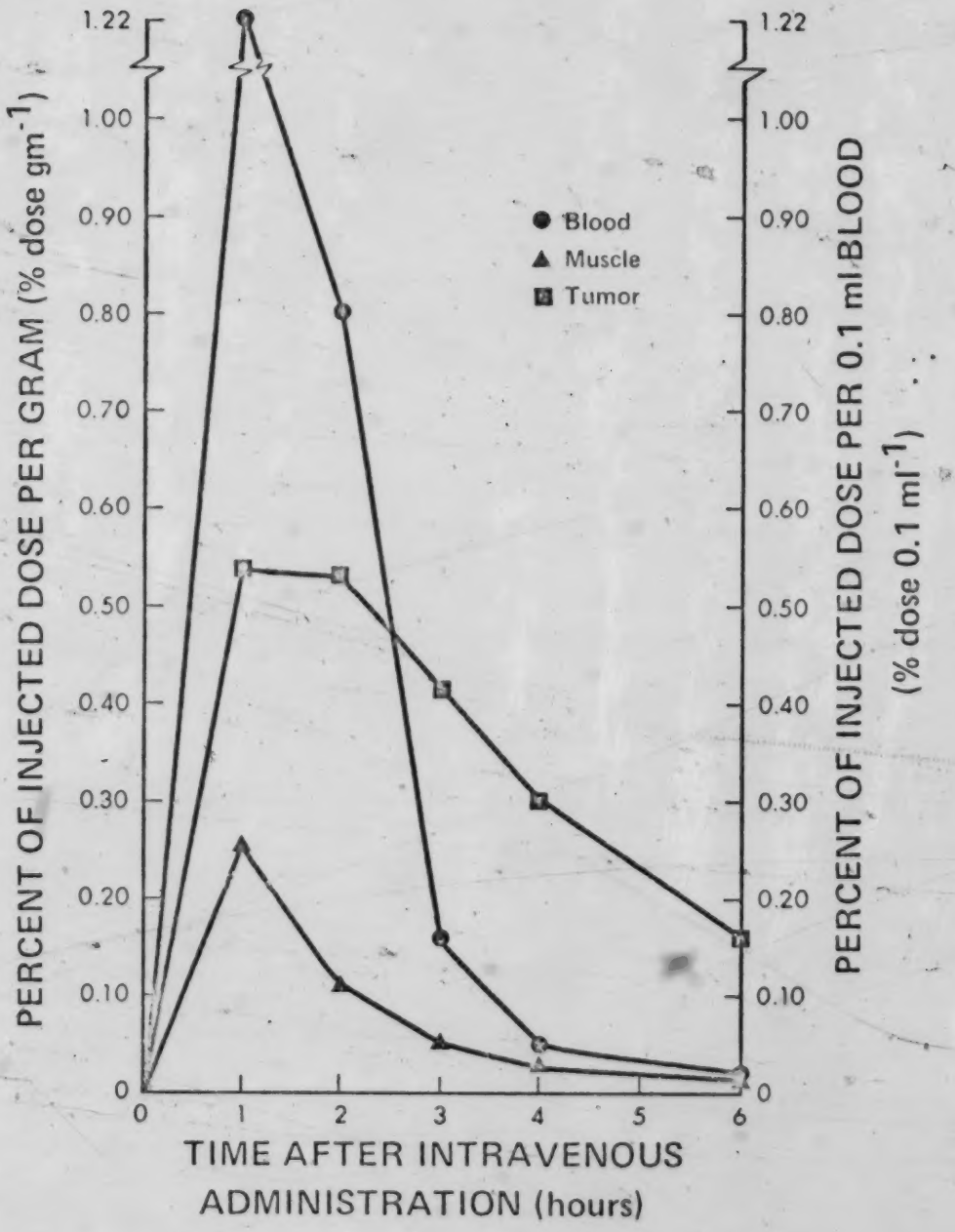


Figure 18

Blood and tissue samples were obtained in an identical manner as in previous studies, weighed and transferred to glass counting vials. The results of the percent dose per gram remaining in the samples after gamma spectroscopy assay are shown in table 13 and figures 19 and 20. A high initial radioactive uptake is noted in lung and testes as well as the kidney, blood and tumor but this is rapidly cleared by three hours post-injection of the ^{125}I-bleomycin A_2. Residual high levels remain in the kidney and tumor, and maximum tumor:muscle and tumor:blood of ratios 19:1 and 9.5:1 respectively occur at six hours. As in the normal animal studies the kidney retained approximately 0.5% of the injected dose per gram six hours post-administration.

Again there was no significant difference exhibited between the radioiodinated bleomycin complex and A_2 when the data was subjected to a t-test.

The results of these studies tend to agree with the recent findings of Eckelman et al.[97] His study on tumor bearing rats using different sub-fractions chelated with ^{57}Co indicated that bleomycin A_2 and B_2 are distributed biologically in an almost identical manner. As the bleomycin complex consists of about 90% of these two components it is expected that the complex and the bleomycin A_2 would be comparable in distribution.

F. Tissue Distribution of ^{99m}Tc bleomycin in Tumor Bearing Mice

Bleomycin complex was labeled with ^{99m}Tc using the pyrophosphate reduction method of Bardy.[97] Aliquots of 0.1 ml containing 650 µCi of this solution were injected intravenously into the tumor bearing mice.

Table 13

Tissue Distribution and Uptake of ^{125}I-Bleomycin A_2

in Male IRC Tumor-Model Mice (a)(b)

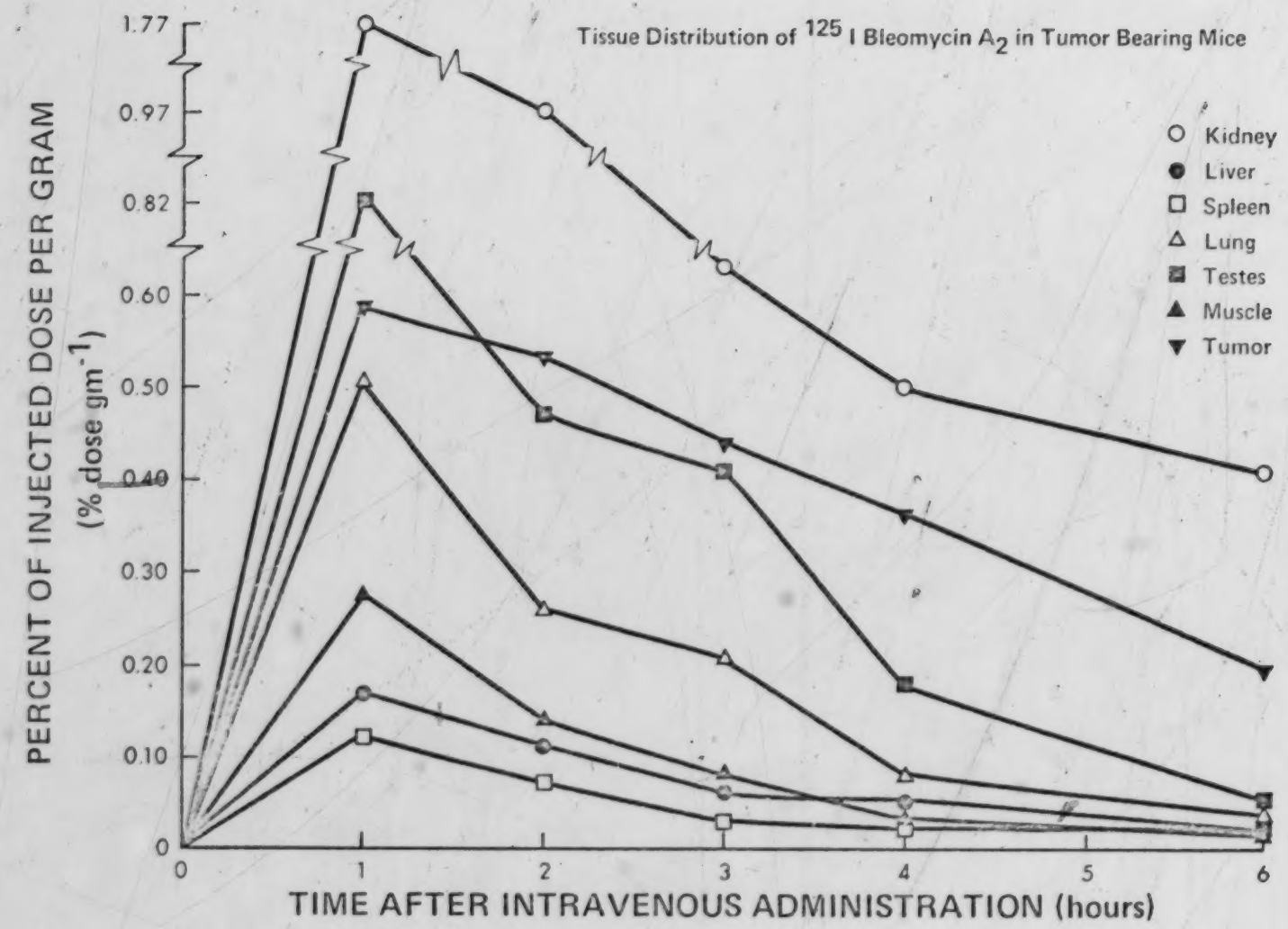
Tissue	Time Post Intravenous Administration				
	1	2	3	4	6
Liver	0.17 ± 0.03	0.11 ± 0.03	0.06 ± 0.01	0. ± 0.0	0.02 ± 0.0
Spleen	0.12 ± 0.04	0.07 ± 0.02	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.0
Lung	0.51 ± 0.03	0.26 ± 0.04	0.21 ± 0.02	0.08 ± 0.01	0.03 ± 0.0
Testes	0.82 ± 0.08	0.47 ± 0.03	0.41 ± 0.02	0.18 ± 0.01	0.05 ± 0.01
Kidney	1.77 ± 0.22	0.97 ± 0.04	0.63 ± 0.01	0.50 ± 0.02	0.41 ± 0.02
Muscle	0.28 ± 0.01	0.14 ± 0.02	0.08 ± 0.01	0.03 ± 0.0	0.01 ± 0.00
Tumor	0.59 ± 0.06	0.53 ± 0.04	0.44 ± 0.02	0.36 ± 0.04	0.19 ± 0.01
Blood (c)	129. ± 0.09	0.83 ± 0.07	0.26 ± 0.02	0.08 ± 0.01	0.02 ± 0.01

(a) Mean of six animals ± S.D. at each time interval and expressed as percent dose per gram of tissue sample.

(b) Each animal was injected with 14 µCi of ^{125}I-bleomycin A_2

(c) Expressed as percent dose per 0.1 ml of whole fresh blood

Figure 19


Tissue Distribution of ^{125}I Bleomycin A_2 in Tumor Bearing Mice
PERCENT OF INJECTED DOSE PER GRAM
(% dose gm^{-1})
1.77
0.97
0.82
0.60
0.50
0.40
0.30
0.20
0.10
0
0
1
2
3
4
5
6
TIME AFTER INTRAVENOUS ADMINISTRATION (hours)
Kidney
Liver
Spleen
Lung
Testes
Muscle
Tumor

Relative Concentrations of ^{125}I - Bleomycin A_2 in Tumor, Muscle and Blood

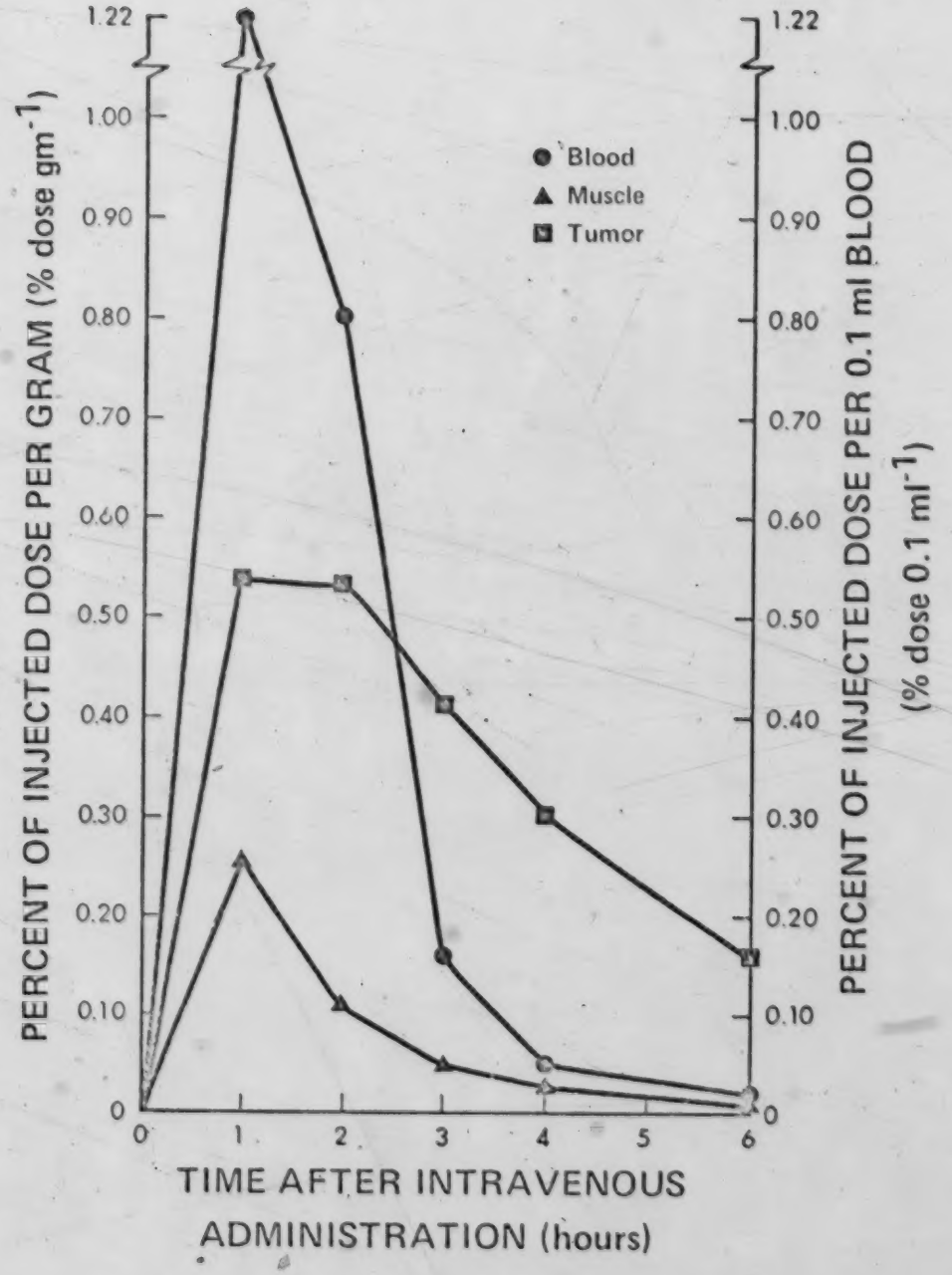


Figure 20

Groups of six animals were sacrificed at the same predetermined time intervals as followed in the previous studies. The various tissue samples were removed and assayed for radioactivity on an appropriately calibrated gamma spectrometer. All counts were corrected for physical decay. The results illustrated in figure 21 and table 14 of this comparative study indicate a more rapid and higher level of uptake than observed with the radioiodinated bleomycins. The very rapid clearance from the tissues with the exception of the kidney is also quite noticeable. The high levels of radioactivity persisted in the blood up to four hours post-administration of the ^{99m}Tc-bleomycin and paralleled the tumor levels. At the six hour time interval the tumor:blood and tumor:muscle ratio was 3:1. These results are in agreement with those obtained by Lin et al.[7]

As shown in table 15 the tumor:muscle and tumor:blood ratios were consistently higher with the radioiodinated bleomycins than with the ^{99m}Tc labeled product. The elevated and prolonged radioactivity values of the blood exhibited in the ^{99m}Tc-bleomycin study would substantiate Thakur's[6] results that the ^{99m}Tc was being metabolically dissociated from the bleomycin molecule and binding to the albumin.

De Nardo's[51 52] initial tissue distribution studies using ^{131}I and ^{123}I iodobleomycin complex yielded tumor:muscle and tumor:blood ratios of 36:1 and 12:1 respectively. The specific activity of the radioiodinated bleomycin was in excess of 100 mCi/mg. The present study of this thesis utilized specific activities of no more than 500 μCi/mg for the animal studies.

Table 14

Tissue Distribution and Uptake of ^{99m}Tc Bleomycin Complex

in Male IRC Tumor-Model Mice (a)(b)(c)

Tissue	Time Post Intravenous Administration				
	1	2	3	4	6
Liver	0.46 ± 0.03	0.31 ± 0.02	0.15 ± 0.04	0.06 ± 0.02	0.02 ± 0.01
Spleen	0.21 ± 0.02	0.11 ± 0.01	0.06 ± 0.02	0.05 ± 0.01	0.03 ± 0.0
Lung	0.34 ± 0.02	0.16 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.0
Testes	0.40 ± 0.11	0.14 ± 0.02	0.05 ± 0.01	0.03 ± 0.0	0.02 ± 0.01
Kidney	2.51 ± 0.36	1.72 ± 0.21	1.26 ± 0.26	1.21 ± 0.10	1.08 ± 0.17
Muscle	0.20 ± 0.01	0.14 ± 0.02	0.07 ± 0.02	0.07 ± 0.01	0.03 ± 0.01
Tumor	0.76 ± 0.02	0.41 ± 0.06	0.31 ± 0.02	0.12 ± 0.02	0.09 ± 0.01
Blood (d)	0.96 ± 0.06	0.62 ± 0.08	0.27 ± 0.03	0.08 ± 0.01	0.03 ± 0.01

(a) All counts were corrected for physical decay.

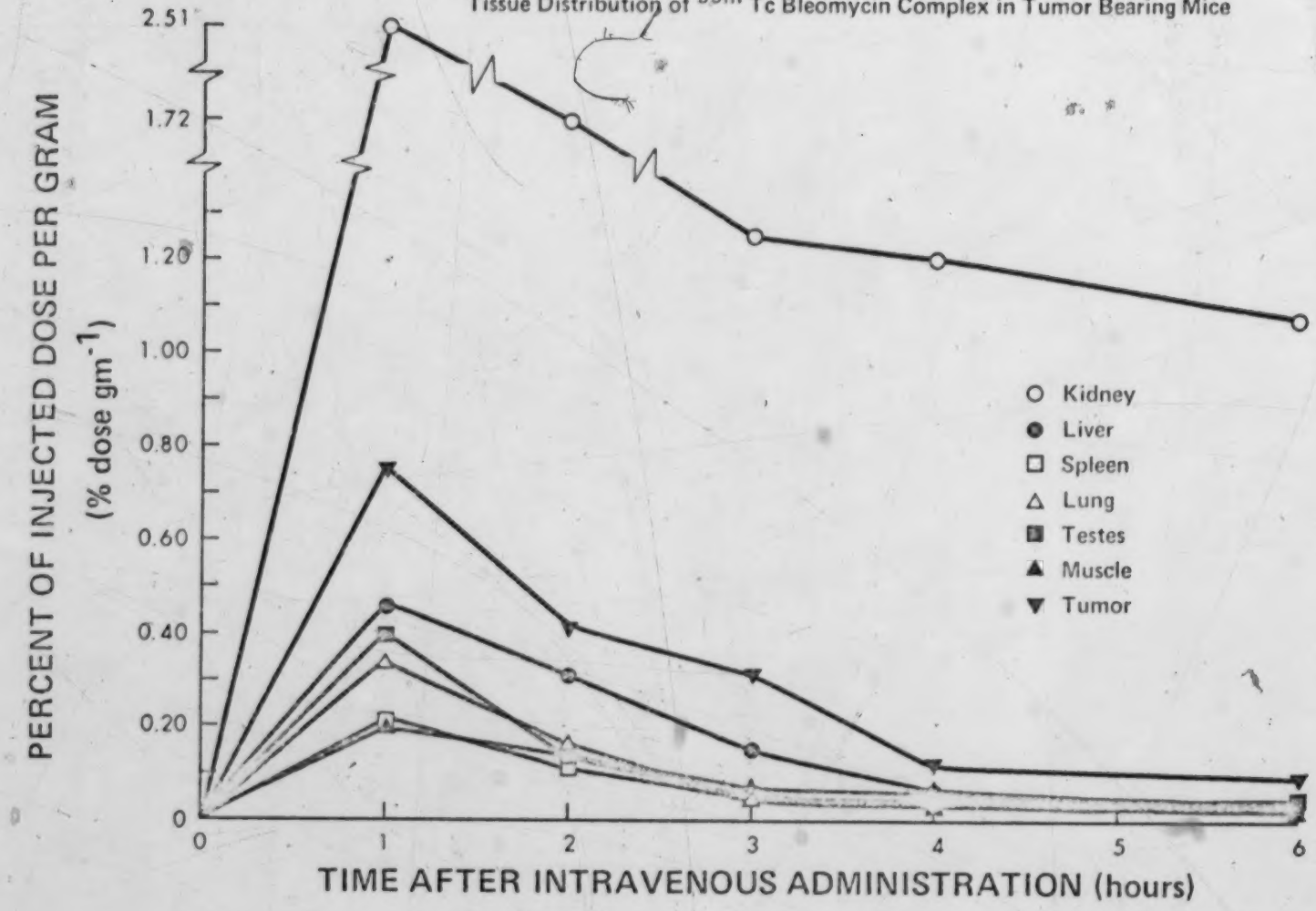
(b) All values are expressed as percent dose per gram of tissue and are the mean of six animals ± S.D. at each time interval.

(c) Each animal was injected with 650 µCi of ^{99m}Tc Bleomycin complex.

(d) Blood was expressed as percent dose per 0.1 ml of fresh whole blood.

Figure 21

Tissue Distribution of ^{99m}Tc Bleomycin Complex in Tumor Bearing Mice



Relative Concentrations of ^{99m}Tc - Bleomycin Complex in Tumor, Muscle and Blood

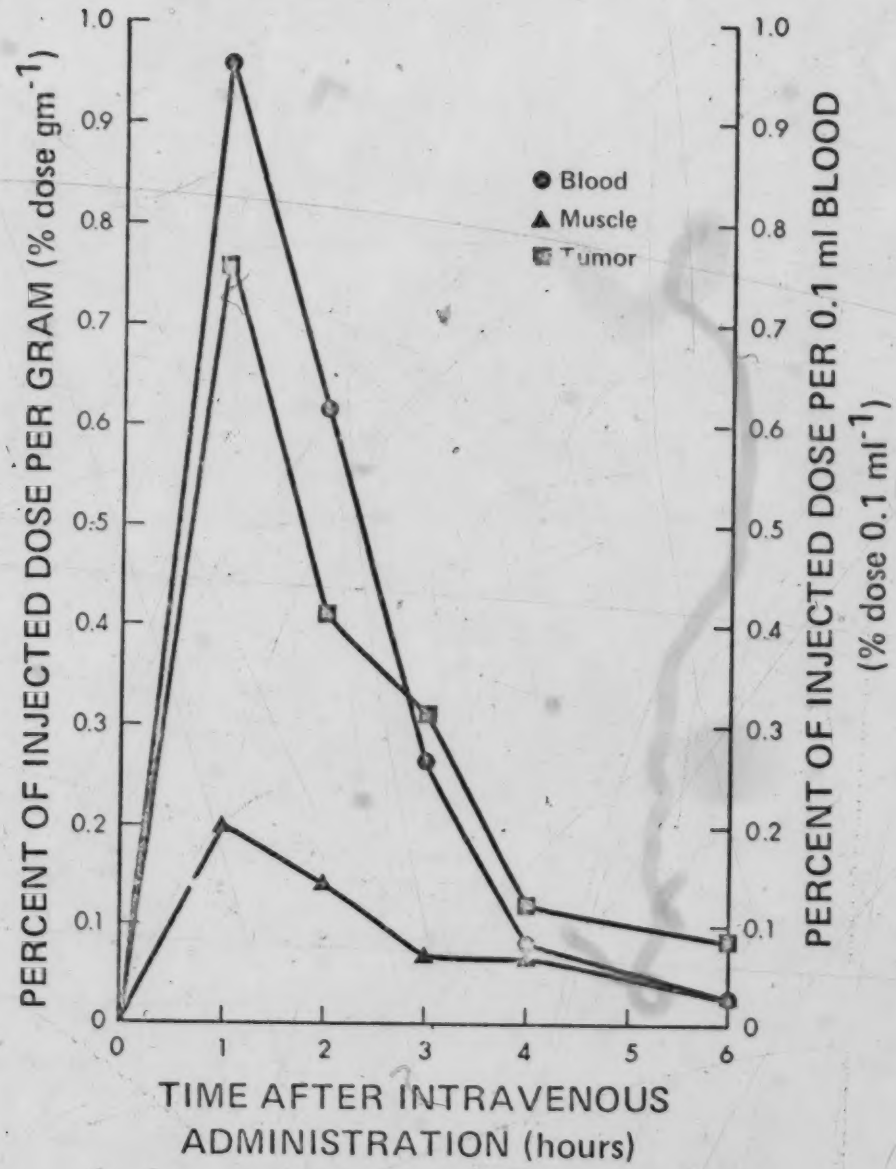


Table 15

Comparative Tumor:Muscle and Tumor:Blood Ratios of ^{125}I-bleomycin A_2, ^{125}I-bleomycin complex and ^{99m}Tc-bleomycin Complex in Tumor Bearing Mice (a)(b)

Ratio	Time Post Administration (Hrs)				
Tumor:Muscle	1	2	3	4	6
^{125}I-bleomycin A_2	2.1	3.8	5.5	12	19
^{125}I-bleomycin complex	2.1	4.8	8.2	10	16
^{99m}Tc-bleomycin complex	3.8	2.9	4.4	1.7	3
Tumor:Blood					
^{125}I-bleomycin A_2	0.45	.64	1.7	4.5	9.5
^{125}I-bleomycin complex	0.44	.66	2.6	6	8
^{99m}Tc-bleomycin complex	0.8	.66	1.2	1.5	3

(a) Expressed as % injected dose gm^{-1} of tumor tissue compared to % injected dose gm^{-1} muscle tissue or % injected dose 0.1 ml^{-1} whole blood.

(b) Mean of six animals ± S.D. at each time interval.

A very recent abstract by Kuschner and Ice[98] reports the effects of specific activity on the tissue localization of various radiopharmaceuticals. As the specific activity was increased the target to nontarget ratios increased and these workers suggest that a direct relationship exists between the administered mass and the resultant target concentrations. Therefore, as the binding sites are saturated maximum uptake occurs and excess is spilled over to nontarget areas. The low specific activity used in this study would contain a large portion of non-radioactive bleomycin which would occupy available binding sites in target tissues and decrease the relative binding of radioiodinated molecules. This factor would tend to lower the radioactivity levels in specific target tissues such as in the tumor and possibly elevate the radioactivity levels in secondary sites. As bleomycin has an affinity for proliferative tissue the secondary or nontarget site would probably be the gonads and would explain high testicular radioactive uptake in some of the studies conducted.

G. Whole Body Excretion

A dose of 15 μCi of ^{125}I-iodobleomycin A_2 in a volume of 0.1 ml was administered intravenously to three mice. Chromatographic analysis of the ^{125}I-iodobleomycin indicated that 96.5% of the radioactivity was associated with the bleomycin and thus 3.5% or .525 μCi of the dose existed as free Na^{125}I.

Ten minutes after administration the total whole body radioactivity was determined in a small whole body counting device. The

count obtained was considered as the initial 100% radioactivity for the individual mouse and all subsequent values were corrected for physical decay and expressed as a precent of this figure as shown in table 16. The whole body radioactivity was determined at time intervals of one, six, twelve and twenty-four hours and then daily for ten days.

The radioactivity was cleared rapidly from the body with over 83% being excreted in forty-eight hours post-administration. Although attempts were made to induce urination prior to assay, residual urine in the bladder of the mice as well as external radioactivity on the fur, may contribute to elevated whole body radioactivity levels.

An excretion curve was constructed and the two components resolved by extrapolation of the linear segments to the appropriate axis. The rapid component was found to have a half life of slightly more than one half day and the slower component had a half life of six and one half days.

These results confirm the in vivo stability of the ^{125}I-iodo-bleomycin. If metabolic dehalogenation was occurring the slow component would be increased considerably due to free ^{125}I retained by the thyroid.

Table 16

Excretion Rate and Total Body Burden of ^{125}I

Following Intravenous Administration

of ^{125}I-iodobleomycin A_2 (a)(b)

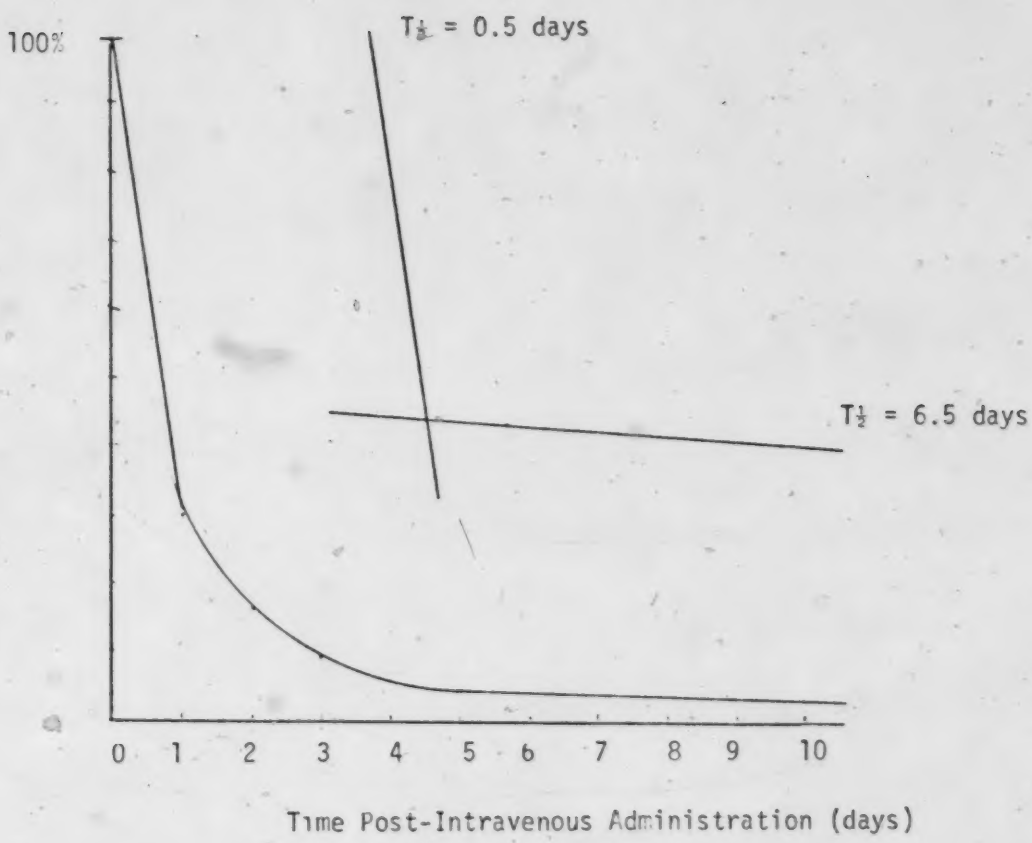
Time Post-Administration (Days)	Percent of Radioactivity Remaining (c)
0	
10 min	100.0 %
60 min	93.9 %
6 hours	74.6 %
12 hours	54.1 %
1	30.9 %
2	16.8 %
3	9.0 %
4	6.6 %
5	3.4 %
6	2.5 %
7	2.0 %
8	1.7 %
9	1.7 %
10	1.3 %

(a) 15 µCi/0.1 ml

(b) Determined by whole body counting and counts corrected for physical decay.

(c) Mean of 3 animals ± S.D.

Figure 23



Compartmental Distribution of Whole Body Retention Curve of ^{125}I After Administration of ^{125}I-bleomycin A_2

SUMMARY AND CONCLUSIONS

1) A chromatographic technique was developed to effectively isolate and purify bleomycin A_2 sub-fraction from the commercially available complex (Blenoxane). An overall yield of 35.7% was obtained from the process.

2) Utilizing an electrolytic cell, bleomycin complex or A_2 sub-fraction could be radioiodinated to predictable yield levels of up to 91%. Chromatographic analysis of the resultant ^{125}I-iodo-bleomycins indicated no gross changes in their migrational behaviour, suggesting that the molecule was not adversely affected under the iodination conditions.

3) A rapid and efficient method, utilizing Gelman ITLC chromatographic material and 95% ethanol as the solvent was developed for assaying reaction progress and radiochemical purity. This technique yielded results that were consistent with the other more conventional methods in one-tenth the time.

4) A final purification technique using a 2 mm bead bed of Dowex 1 x 4 anion exchange resin effectively removed any unbound $Na^{125}I$ from the terminal iodination reaction mixture. The resultant radiochemical purity of the ^{125}I-iodobleomycins after this treatment was in excess of 95%.

5) It was found that radioiodinated bleomycins were stable at 4°C and at room temperature, dehalogenating at the rate of only 0.7% and 1% per day. The carbon-iodine bond remained intact in the presence of Cu^{++} ions and a loss of 12% of the radiolabel was

observed when ^{125}I-iodobleomycins were exposed to 100°C for sixty minutes.

6) Tissue distribution of the ^{125}I-bleomycin complex of ^{125}I-bleomycin A_2 sub-fraction showed no significant differences in either normal or tumor-bearing mice. Uptake of both compounds was rapid in all tissues but had cleared noticeably by six hours post-administration with the exception of the kidney and tumor. The residual radioactivity in the tumor yielded maximum tumor: blood and tumor:muscle ratios of 9.5:1 and 19:1 respectively for the ^{125}I-iodobleomycin A_2 and 8:1 and 16:1 respectively for the ^{125}I-iodobleomycin complex at six hours post-injection.

7) Comparative tissue distribution studies with ^{99m}Tc-bleomycin complex showed rapid and high initial uptake of the radioactivity with a subsequent rapid clearance from all tissues with the exception of the blood, kidney and tumor. High blood radioactivity levels persisted and yielded a maximum tumor:blood ratio of only 3:1 at the six hour time interval. The tumor: muscle ratio at this time was also 3:1.

8. Excretion of the ^{125}I-iodobleomycins from mice was rapid with only 16.8% of the administered dose detected by whole body counting after forty-eight hours. A whole body excretion curve indicates two components, a rapid segment with a half-life of one half day and a slower segment of six and one half days.

From the results of this project it can be concluded that the electrolytic radioiodination of the bleomycin complex produces a viable tumor localizing agent that is stable *in vitro* and *in vivo*. If ^{123}I was utilized as the covalently bonded nuclide, scintigraphic imaging could be conducted with the present scintillation detection cameras as an aid in the localization and detection of various malignant tissues. Due to the high extraction cost for isolating the pure A_2 sub-fraction and the apparent near identical tissue distribution pattern when compared to the total bleomycin complex, a practical radiopharmaceutical could be produced by radioiodination of the commercially available bleomycin complex. While the electrolytic technique is not as rapid as other iodination methods, the high, predictable yields make it the method of choice if reaction time is not a factor.

The introduction of the Gelman ITLC quality control system has provided an extremely rapid method of testing radiochemical purity. A total of ten minutes was all that was required for complete chromatographic assay and analysis using this system.

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